

## What should the young be told?

*Britain's planned revolution of school education is potentially both rewarding and disastrous. The government had better go carefully.*

THE post-election British government has embarked on a remarkable task — the design and enforcement of a national curriculum for secondary schools. Bucking its own trend of letting things find their own level, preferably a market-level, it plans in the autumn to decree that the education of the young should be built around a small set of examinable bodies of knowledge. The proposals will certainly become reality; too many British parents are vaguely dissatisfied with the education of their children for it to be otherwise. The danger is that the British government, in forcing through the House of Commons 500 clauses, each of which might occupy a parliamentary session, will do a disservice to its own people and set a bad example for other governments.

There is nothing wrong with the notion of a core curriculum — indeed, the opposite is true. Plainly it would assist the mobility of families from one place to another if there were some general understanding of what young people should be taught at school. There may also be something in the British government's claim last week that a national curriculum would enable parents more accurately to tell to which schools they should send their children, although that contention will occupy much of the parliamentary time between now and the next-but-one parliamentary session, if only because of the suspicion that parental choice is a euphemism for privately financed education. Certainly it would help if young people themselves had some general idea of the objectives of school education. Is it cultural, or for helping to find a job? (Or does the second follow from the first?) The upbeat message in the government's own justification of its intended course of action is that too many young people's expectations of themselves are at present deflated by their teachers' diminished expectations of young people.

The snags, as always, come in the fine print. On the face of things, the proposals in the British government's consultative document are reasonable enough: let there be "maths" (for mathematics), English and science at the core of the core; beyond that will follow "a modern foreign language, technology, history, geography, art, music and physical education". As in all British schools since 1944, there will also be a modicum of religious education. An enchanting block of fine print dealing with the teaching of the Welsh language carefully avoids the issue of whether this minority language will be an acceptable alternative to English. But, for the generality of British students, there will be test of attainment in the core-core subjects every few years (at ages seven, eleven, fourteen and sixteen). The intention is that the results of these attainment tests should be public knowledge so that parents may choose between schools and students be catered for appropriately.

### Danger

Superficially, there is nothing wrong with what is planned, but there is a danger that the sum of human knowledge has been so whittled down, in the British government's proposals, that when used not merely as a test of individual students' attainments, but of those of schools, it will be a self-defeating goal. Take "maths", for example. If a school knows that its public reputation will hang on its overall attainment at pre-determined

aptitude tests, will it ever make sense for it to allow teachers occasionally to teach Latin to odd-ball students when they might be more gainfully employed drilling young people in the mysteries of proportionality? The risk that the core curriculum will be homogenized pap needs to be considered.

There is also a problem about science, trendily part of the "core of the core" because of the British government's touching (but correct) devotion to the belief that science will breed technology that will breed prosperity. The fallacy in the inference that everybody should be taught science from the year dot is most simply exposed by the simple question of how many captains of industry admit to having been persuaded that the future would be both more technological and more full of opportunity because of what they had been taught of science at their primary schools. The plain truth is that there is no known successful way of giving very young people a sense of science and technology, but only a smattering of a sense of what they may be about. Some very young people with intellectual energy to spare find out, usually by accidents that clever teachers devise at later ages. In Britain in particular, there is a depressing record of how too much formal teaching of science (and even mathematics) has driven bright young people in the opposite direction. Can it be wise to engineer a revolution in the British educational system when a third of the assessable content of the curriculum is unteachable between the ages of, say, 5 and 12? Would not the same young people be better occupied learning French, or German, or even Welsh?

### Dispute

It would be a less taxing conundrum if there were different teachers. Conventional wisdom has it that British schools have gone to the dogs because the teachers are no good, but that is only a reflection of the way in which British teachers have appeared recalcitrant employees of the public service in the past three years of dispute with their employers (essentially the central government) over pay. The government has won that battle, but may have lost the war by demoralizing the people on whom it must depend, not merely to make the core curriculum a reality, but to enrich the prescription now on offer with the ingredients that will allow young people leaving British schools to believe themselves to have been educated for the modern world. To put things right and to make its core curriculum a success, the government will have to pay out more than the extra £3,000 million a year it found on the eve of the general election to settle its pay dispute with the teachers to give the teachers the belief that their schools are again properly equipped.

The British government's problem, which falls to Mr Kenneth Baker, its Secretary of State for Education and Science, is that of turning its largely dedicated teaching force into people who believe their students have a brighter future. The fly in that ointment is that the British government appears to be heading in two directions at the same time — towards a national system of publicly maintained schools in which a single curriculum will be supreme and towards an enlarged system of private education to which fewer restraints (except those of parental approbation) will apply. Can that, politically, make sense? □



## Bad advice on AIDS

*President Reagan should counter the defects of his new AIDS panel by risking his own popularity.*

How should a government go about seeking advice on a difficult problem that threatens its people's public health? One way, the bad way, is to find a group of people who subscribe to the government's ideology, paying little or no attention to the expertise they may possess. The recommendations of this panel can be counted on to be "correct" in that they will conform to the government's own views of the world, but they would not be likely to break new ground. Another way, for the sake of argument "the good way", is to assemble people with genuine and first-hand knowledge of the problem, whose preconceptions, in the nature of things, are likely to be divergent opinions about how it should be approached. Such a group is certain to present its sponsoring government with difficult choices, some of which would certainly fly in the face of its ideology. But such a group might at least also suggest creative and effective solutions, leaving political and ideological problems to others better equipped to handle them.

President Ronald Reagan, faced with the need to decide how the United States should respond to the problem of AIDS (acquired immune deficiency syndrome), has chosen the bad way (see page 372). His advisory panel consists of people whose ideological qualifications are clear, but whose expertise on AIDS is not just suspect but non-existent. Theresa Crenshaw, the sex therapist from San Diego, supports abstinence from sexual activity as a general prophylactic against AIDS. That is symptomatic of a philosophy already espoused by the Reagan Administration for "wrong" behaviour of which it disapproves: "Just say no." This approach has not worked for drug abusers, and will not control the spread of HIV infection.

AIDS presents special problems for a country such as the United States which is uncomfortable dealing with issues relating to sexual behaviour. It is ironic that a president as popular as Mr Reagan is probably the only person who could openly tell the United States, without alienating his listeners, that AIDS is a particularly insidious (and dangerous) often-venereal disease whose spread will be successfully controlled only if people give thought to the nature and the safety of their sexual practices. If, for example, he were to tell the United States that people at risk should be careful to use condoms, there is a chance that they would believe him. But Mr Reagan will not take that chance. Although only a small fraction of those suffering from AIDS in the United States are children — homosexuals are still the majority — the president, during his visit to the National Institutes of Health last week, chose to have his photograph taken last week with a black haemophiliac child suffering from AIDS. The gesture may have helped to convince some who saw it that HIV is not an airborne infection like the common cold, but it will have done nothing to carry home to US citizens the stark truth that AIDS is laying waste to an identifiable and substantial part of the population of the United States. Is it that he would prefer not to acknowledge, or to be seen by his supporters to acknowledge, that homosexuals exist?

The pity in all this is that there is little doubt what needs most urgently to be done. Last year's excellent report from the National Academy of Sciences and the Institute of Medicine made it crystal-clear that explicit public education is an essential first step. Getting at young adults would require mass advertising not very different from that which has startled (and sometimes shocked) people in places as different as Australia and Britain, in the process acknowledging that many modern practices now common — pre-marital intercourse and drug abuse as well as homosexuality — do not easily fit in with the mores of polite conventional society. Beyond public education, there are stronger nettles to be grasped, questions of the balance between personal liberty and communal health, that will be

tackled wisely only by well-prepared communities. Yet these are the issues for which the US government needs advice. The panel now appointed will deliver nothing of the sort. President Reagan may know that in advance. Meanwhile, he could do worse than use his popularity to force open the tight moral lid that his conservative advisors would like to see placed on the problem of how to live with and, with good judgement, to survive AIDS. □

## Jumbos in the skies

*The planned merger between Britain's two largest airlines raises questions of principle.*

AIR transport has for decades deserved better from the governments that regulate its affairs, or otherwise interfere. So much is plain in Europe, where a network of state-subsidized airlines still runs a series of cartels to keep the cost of air travel at outrageously inflated levels. Only two national airlines have stood out against these practices — British Airways and the Royal Dutch Airline KLM. Now, under pressure from the European Commission to operate more competitively, European airlines have taken to pointing to the United States, where airlines were deregulated five years ago, saying that experience shows that competition does not work. Smugly, they note that many of the new airlines founded in the wake of deregulation have since been swallowed up by larger competitors; they go on to imply that Europe would not wish to be bothered with that inconvenience. What they overlook, no doubt deliberately, is that the airlines surviving in the United States are more efficient than their progenitors — and that, if they are not, newcomers retain the deregulated right to compete with them.

The next few weeks should bring an interesting test of the cosy European system. British Airways, by far the largest British carrier, announced last week that it plans to buy its largest British competitor, British Caledonian. The explanation is no secret. British Caledonian has been complaining for years that, so long as the British government's regulatory body, the Civil Aviation Authority (CAA), continued to refuse it the right to fly enough routes to be economically viable, it would have no choice but to merge with British Airways.

What this sad tale reveals is the sheer arbitrariness of the regulatory process, even when some of those concerned profess themselves opposed to the cartelization of European air transport. (The British government has been as stalwart as British Airways in its opposition to the European ramp.) A licence to operate aircraft along a commercial route may not be a licence to print money, but it can be a useful defence against bankruptcy. CAA could obviously have helped to keep British Caledonian in the air by giving it access to a larger piece of the cake, the total size of which is determined by bilateral negotiation between European governments, but only at the expense of British Airways, which was sold five months ago to the British public in a hugely successful share flotation. Can it be right that a quasi-judicial regulatory agency should have the incidental power to determine the fates of companies into which private people have sunk their funds?

For British Airways (and its competitors), the interest now will be whether the planned merger will be allowed (subject to the approval of the Mergers and Monopolies Commission and/or the British government) to go ahead. That British Caledonian will retain its name and its existing routes must reflect a calculation that if the merger were a simple takeover, the routes would be up for grabs again. For European air travellers, the chief interest will be whether British Airways, protestations of its zeal for competition have survived the transition from nationalized industry to private company. The British government could and should put the issue to the test by saying that if the merger is allowed, Britain's other small airlines, perhaps half a dozen at the most, will be allowed to compete freely with the surviving giant on any routes to which they can wheedle access. □



# Japan broadly agrees to participation in SDI

- Senate approval remains uncertain
- Japanese fear restrictions on commerce

## Tokyo & Washington

JAPAN and the United States last week signed an agreement opening the way for Japanese companies and government research laboratories to join in the research phase of the Strategic Defense Initiative (SDI). But Japanese participation may be limited, both because of reluctance by Japanese industry to join in the project and efforts by the Congress to keep research dollars inside the United States.

It is more than two years since US Secretary of Defense Caspar Weinberger invited Japan to join the SDI research programme. Prime Minister Yasuhiro Nakasone, although keen for Japan to participate, proceeded very cautiously for fear of rousing protest from the opposition parties and the public. But apart from token resistance by scientists, opposition failed to materialize and, in September last year, Japan accepted Weinberger's invitation.

Since then, Foreign Ministry officials in Tokyo have been learning what is involved in negotiating contracts with the Pentagon, something new to Japan, while trying to secure terms favourable for participating Japanese industries. Many companies are interested in SDI research because of possible commercial spin-off, but fear that US secrecy and patent restrictions might rob them of any benefits gained by joining the programme.

Some industry fears will be allayed by the parts of the agreement made public. No restrictions will be placed on technology developed by Japanese companies before their involvement in SDI research and, although patents arising from the research will be held by the United States, companies may license the technology free of charge. But the technology may be used commercially only if it is unclassified and, according to a Foreign Ministry official who helped to negotiate the agreement, the Pentagon has "full authority to classify what it chooses".

Even if Japanese companies decide to join, they may find their way blocked by the US Congress. The House of Representatives has already passed an amendment to the defence authorization bill that would prohibit foreign participation in SDI research unless the work cannot be "competently performed in the United States" for the same money.

If the Senate adopts the same language, some participating governments fear that

their own SDI research agreements will be rendered obsolete.

The timing of the Japanese agreement is significant, coming as it does on the heels of the Toshiba incident — the illegal export of milling machines to the Soviet Union by Toshiba Machine Co. (see *Nature* 328, 283; 1987). There were widespread reports of the case in the United States just as Congress was about to consider legislation liberalizing export controls. Congress will soon take the final steps to send the legislation to President Reagan, who has threatened to veto it.

The Toshiba case has brought on a bout of "Japan bashing" by the Congress, with sabre-rattling legislative sanctions and the public smashing of a Toshiba cassette-player with mallets. On the other hand, the Toshiba affair has allowed the Pentagon and the Nakasone administration to insist on increased Japanese-US collaboration in technology transfer, and a clamp-down on COCOM violators.

Japan's ruling party intends to revise and/or renew the Foreign Exchange and Foreign Trade Control Law to tighten controls on high-technology exports to communist countries, and to treble the staff at the Ministry of International Trade and Industry (MITI) responsible for screening exports. In addition, MITI will draw up strict rules to protect military secrets from being given away by Japanese companies participating in SDI research. Under normal circumstances, such moves to strengthen government control of secrets would have raised howls of protest in Japan. In the wake of the Toshiba incident, however, they will probably pass unopposed.

But, considering the treatment of Toshiba by Congress, will Japanese companies still want to participate in SDI research? At least five or six companies are interested and have carried out in-depth studies, says one of the Foreign Ministry negotiators. Even Toshiba's new president, Joichi Aoi, says his company still has ambitions to participate.

Other electronic producers holding technological advantages over the United States in such areas as gallium arsenide and laser diode research — the very areas of most interest to the Pentagon — still fear that their technology may be classified as secret if they join. Developments over Toshiba are doing nothing to ease their concern.

David Swinbanks & Joseph Palca

# India builds big radiotelescope

## New Delhi

INDIAN astronomy is looking up after government approval for building what is likely to be the biggest radiotelescope of its kind in the world. It will observe metre-wavelength radiation from the Universe, a region of the electromagnetic spectrum blotted out from telescopes of the Western world due to manmade radio interference.

The \$20-million Giant Metre Wavelength Radio Telescope (GMRT) will be built at Narayangav near Pune, which is about 200 km east of Bombay and free from pollution. The telescope, expected to be operational in 1992, will operate at frequencies of 38–150 MHz and 325 MHz and 610 MHz and will be designed and built almost entirely by Indians.

"GMRT is a marriage of the world's two big radiotelescopes — the Very Large Array in New Mexico and the Arecibo radiotelescope at Puerto Rico — with the advantages of both", says Govind Swarup, director of the radioastronomy centre at the Tata Institute of Fundamental Research (TIFR) and the brains behind the project. The telescope will consist of 34 identical, steerable, 45-m diameter parabolic dish antennae, forming a Y-shaped configuration spread over an area of 25 km<sup>2</sup>. To reduce weight and cost, the giant antennae will have 'see-through' wire-mesh reflecting surfaces instead of solid steel.

According to Swarup, GMRT will be capable of resolving radio sources up to a few arc-seconds and will also provide high-resolution maps of galactic and extragalactic radio sources. GMRT will be open to all astronomers in India as well as to those elsewhere. Swarup expects that 10 per cent of the operational time of GMRT would be allocated for the best research proposals from astronomers from abroad.

GMRT will enable India to enter the high-technology astronomy race, but there is concern about the lack of a sufficient number of astronomers in India because very few Indian universities offer degrees in astronomy, which in turn is due to limited facilities in India for doing research in astronomy.

To solve the manpower shortage, a scheme has been launched jointly by TIFR and the University of Pune under which selected students will be given scholarships to work for master's degrees and doctorates in astronomy. It is also planned to advertise for scientific manpower in the United States where a number of Indians are studying astronomy or doing postdoctoral work.

K.S. Jayaraman



# Koop's corps of biomedical workers cured with kindness

Washington

POLITICAL struggles within the US Department of Health and Human Services (HHS) may soon yield a surprise reward for biomedical researchers working at such laboratories as the National Institutes of Health (NIH). An HHS plan for a new "senior biomedical research service" would raise top salaries to \$110,000 a year, making scientists better off than senior government officials, including even cabinet secretaries.

According to HHS officials a rise is needed to stem the drain of top researchers out of their laboratories and into the universities and industry. Clinical department chairmen in medical schools now average \$167,000 a year, almost double the top pay of \$85,000 a year at NIH. But the existence of the salary gap does not completely explain HHS's interest in a new research service; it may have much to do with a separate issue, the wish of Surgeon General C. Everett Koop to revitalize the Public Health Service Commissioned Officers Corps.

The corps is no longer a highly visible body and many of its functions have passed into history (see right). Over the past twenty years it has often been under threat of abolition even though the Surgeon General, the corps' chief officer, has traditionally functioned as an outspoken guardian of the nation's health.

Koop is no exception to the role and has weighed in with controversial pronouncements on AIDS. He also proudly wears the corps' uniform and has announced his determination to revitalize its spirit. Unless a major role is found for the corps, he says, "we would see its demise within a decade". Koop's solution stresses the military spirit of the corps, "expert, flexible and mobile; our only defence against non-military disaster". As examples of what the corps can do, he cites the emergency work carried out when Cuban refugees arrived in Miami and recent postings to drought-stricken regions of the Sudan.

The corps, as Koop freely admits, is less than mobile and has far too many officers in the upper ranks. But not all his first efforts to bring change have been welcome. Attempts to make staff retire after 30 years' service were virtually abandoned after NIH pointed out that some of the most productive research groups would thus be left leaderless. But uniforms are once more being worn.

Opposition to Koop's revitalization

## Correction

The director of the MacArthur Center for Tropical and Parasitic Diseases at the Weizmann Institute in Israel (see *Nature* 327, 581; 1987) is Professor Ruth Arnon. □

plans is strong among senior members of the corps, many of whom joined during the Vietnam war when service offered an alternative to a spell in the army. The pay plan may provide a way around them. At present, corps members are often better paid than civil servants in the same laboratory, with top salaries around the \$100,000 mark. But under the new scheme the same researchers would be better off if they transferred out of the corps — leaving Koop with those who are willing to go along with his ideas.

Whether the scheme will overcome opposition from those anxious to reduce the cost of government remains to be seen. If it is successful it could help both to revitalize the Commissioned Corps, and stop the brain drain from NIH. But it will leave less senior biomedical researchers wondering why they too cannot enjoy salaries of the kind they hear about in universities and industry. Alun Anderson

● Most of the Commissioned Corps' 5,364 members work at the Health Resources and Services Administration, but a large contingent is also found doing research at NIH, where one of its best-known officers is AIDS researcher Robert Gallo. A walk down NIH's corridors would not, however, reveal who is a commissioned officer and who an ordinary civil service employee but in law largely forgotten differences exist.

The Commissioned Corps was created in 1889 when Congress turned the Marine Hospital Service into a military organization with titles and pay corresponding to Army and Navy ranks. Today, the corps is still one of the uniformed services of the United States, alongside Army, Navy, Air Force, Marines and the Coastguard; in time of war or national emergency, the president can designate it a full military service. Rarely applied military-style regulations still exist: its officers can be rotated to new commands at short notice, be required to stand down after 30 years' service, and be required to wear their Navy-style uniforms.

The corps' heyday came perhaps earlier this century when the rapid deployment of skilled health workers was essential to prevent epidemics. In the corps' own manuals, the military spirit still lives on: the officers are soldiers and "in the silent war against disease, there is no truce — no retreat — no peace. The unrelenting battle goes on against the deadly enemy . . . it stalks the world wantonly crippling and killing millions."

Modern functions of the corps include providing health care for native Americans and for isolated communities. But these are perhaps its only unique functions. □

## NET succeeds JET

The European Commission has committed part of its newly-agreed research budget to the construction of the Next European Torus (NET), the successor to JET (Joint European Torus), the research-level machine now operating successfully at Culham in Oxfordshire, Britain, which is also the site of the UK Atomic Energy Authority's thermonuclear fusion laboratory. In total, the commission has allocated ECU 911 million (about £700 million) for NET, on which construction would begin only in the 1990s. The budget is expected formally to be agreed this week at a meeting of Community finance ministers, but decisions about the siting of the enterprise will be made only at a later date. Culham has been mentioned as a suitable location, but West Germany is also a strong contender. K.J.

## Science council change

The British Science and Engineering Council (SERC) is to be reorganized in a way that will give its chairman more influence. After the retirement of the present secretary to SERC, Ashley Catterall, next year, the post will be reduced in importance. The present chairman, Professor W. J. Mitchell on a five-year secondment from the University of Oxford, plans a trichia of administrators — one (the head of the Rutherford-Appleton Laboratory) dealing with research laboratories, another (Dr Harry Atkinson) channelling the opinions of the four research boards to the chairman and yet another administering grant applications and SERC housekeeping. □

## Tenure to go in Britain

Against expectations, the Secretary of State for Education and Science in the British government, Mr Kenneth Baker, has decided to include the abolition of academic tenure in his forthcoming education bill to be published in the autumn. The proposal is certain to be controversial: earlier this week, a spokesman for the Committee of Vice-Chancellors and Principals asked whether [this] is "to cut costs or to cut dissent". Ms Diana Warwick, secretary-general of the Association of University Teachers, says that the objective must be to make universities contract more quickly. Academic tenure features in the standard contracts of employment of only 23 out of 47 universities in Britain, many of which are considering whether their royal charters should be changed. The government's proposals would apply only to newly appointed academics and to those changing institutions, but a novel feature is that a person's "inefficiency" is added to the list of "good causes" for which a person may be dismissed, of which the chief is the institution's inability to pay his/her salary. K.J.

# Muted response to proposals to reform British universities

London

PROPOSALS to change the basis of scientific research and teaching in Britain's higher education institutions have not provoked the full-throated outcry that might have been expected. Academics seem in general to accept that decreasing government support means that the present system of a wide spread of scientific research across many institutions, although desirable, is no longer sustainable. It is widely agreed that a greater level of concentration of research is needed, but serious doubts persist about the wisdom of introducing a formally tiered system in the way that is being recommended by the Advisory Board for the Research Councils (ABRC) in a discussion document released last week (see *Nature* 328, 280; 1987).

ABRC's suggestion is that some institutions should be devoted mainly to undergraduate teaching, others (about 15)

should offer undergraduate and postgraduate teaching and substantial research facilities across the range of fields, and a third category whose teaching and research is across a narrower range of fields.

The main fear expressed by the heads of Britain's universities is that the proposed system would lack flexibility and not be amenable to review: that once categorized, institutions would be unable to 'better' themselves.

The Committee of Vice-Chancellors and Principals (CVCP) is opposed to any formal stratification, although it concedes that the report has much to commend it and that it could have been potentially more damaging. But CVCP is wary of several possible pitfalls, in particular the difficulty of choosing criteria to identify an institution's future status.

Unsurprisingly, it is the smaller universities, with one or perhaps two successful

research departments, that are most fearful of the new system. The heads of such institutions argue that a successful department should not be penalized because the institution as a whole is not deemed suitable to be included in one of the research categories. The department should itself be the focus for related work, and a research centre set up around it. Such a system, however, would be unlikely to be able to support the relatively few interdisciplinary research centres that ABRC is proposing.

Whether the suggested system of 'teaching-only' institutions would be able to carry out the sort of research needed to produce high-calibre graduates is another question that is troubling academics.

Not everyone agrees with the ABRC's proposals. Some vice-chancellors say that the present system of selectivity for funds being adopted by the research councils and the University Grants Committee is perfectly adequate, and that, left alone, a naturally differentiating system would evolve.

Simon Hadlington

● If proposals to reshape British academic science are to be successfully implemented, a substantial increase in investment in the science base is necessary, the Education Secretary, Kenneth Baker, has been told. In its annual advice to the government on the science budget, the Advisory Board for the Research Councils (ABRC) calls for an extra £103 million in 1988–89, rising to £166 million by 1990–91. The budget is currently £676 million. Last year, ABRC requested only an extra £35 million. The size of the request has cheered the science community, but it remains uneasy about ABRC's proposals for a three-tier classification of higher-education institutions.

In its spending advice, ABRC reiterates the urgency of establishing interdisciplinary university research centres, and proposes that six such centres be created in each of the next three years, which would require additional funds of £90 million.

Bids for the first such centre, in high-temperature superconductivity, have already been invited (see p.370). ABRC has identified several research programmes with "potential strategic importance" that require additional funds of £94 million over the next three years. They include a successor to the Alvey programme of research into information technology, biotechnology, radiological protection, environmental microbiology and ocean flux. Further cash is needed, says ABRC, for re-equipping the best research groups, restructuring of the research councils, support for fellowship schemes and compensation for pay settlements.

Ominously, ABRC, is assuming a "marked reduction" in the United Kingdom's subscription to CERN (The European Organisation for Nuclear Research). A final decision on continuing collaboration is expected in the autumn. □

## Syrian cosmonaut reaches space station

London

THE Soviet Union's first guest-cosmonaut from the non-socialist developing world, the Syrian Mohammad Faris, arrived safely aboard *Mir* space station last Friday. The flight plan was somewhat unusual; the Soviet space-planners used the relatively new 48-hour trajectory between launch and docking, which, according to deputy flight controller Viktor Blagov, reduces the strain on both crew and ground-services, and also entails minimal expenditure of energy. Also for the first time, the manned transport craft came in to dock with the space station from the side of the astrophysical module.

Faris's programme aboard *Mir* follows closely the pattern established by previous guest-cosmonauts — a mix of medical, biological and technological work and photography and telemetry of the guest's homeland. In particular, the joint programme includes the production of crystals (gallium arsenide) and a eutectic alloy of aluminium and nickel, to study the

effect of microgravity on structure. But in one respect, Faris's experiments differ from those of the previous 'guest'. These had to do their experimental work in the *Salyut* space station itself, where even during designated rest periods, the cosmonauts' movements could create sufficient vibrations to affect the crystallization process. But in the case of *Mir*, experimental work is not done in the space-station proper, but in one of the specially equipped experimental modules docked with it. As Aleksander Dunaev, head of the new Soviet commercial space agency *Glavkosmos* explained recently, to carry out a technological experiment, the crew load the furnaces and switch on the appropriate programme, and then undock the module. The latter then remains in independent flight until the experiment is complete, undisturbed by any crew activities. At the end of the experiment, the module is docked again with the station, and the experimental capsules can be withdrawn for return to Earth. Vera Rich



The Soviet-Syrian crew: Faris (left), commander A. Viktorenko and engineer A. Alexandrov.



## Competition before cooperation at US superconductor conference

Washington

PRESIDENT Ronald Reagan's administration is taking the promise of the newly discovered high-temperature superconductors seriously. The president himself will give the keynote address at the Federal Conference on Commercial Applications of Superconductivity being held in Washington DC this week steps have also been taken to stop foreign officials and company representatives attending.

The latter move was greeted with some surprise by embassy science officials, who are normally welcome as observers at scientific conferences. Although some have found ways of sneaking staff into the conference, most have been forced to accept that attendance is by invitation only and is intended to help US industry gain an edge in the world-wide race to commercialize the new superconductors. The conference is sponsored by the White House and the Department of Energy.

Whether anyone has "sat down and calculated the costs and benefits of such a policy" is open to doubt according to Robert Parks, director of the Washington office of the American Physical Society. Although major new research results are

not expected to be announced at the conference, and foreign scientists will lose little by not attending, any step that appears to block the free flow of information may eventually work against the United States. An enormous amount of basic research remains to be done and will inevitably be hastened by international cooperation. Key discoveries have already come from Switzerland and Japan.

Despite the ban on attendance by foreign officials, no restrictions have been placed on foreign news reporters. Over 1,000 people, drawn largely from industry and government, are expected to attend the conference. After a brief introduction to the science of superconductivity from the University of Houston's Paul Chu and Nobel-prize winner Robert Schrieffer, they will spend most of their time hearing from government officials about the future applications of superconductors, the technological problems that need to be overcome and "foreign technological advances". One speaker will be missing. The Secretary of Commerce, Malcolm Baldrige, who was due to speak after the President, died after being thrown by a horse at the weekend. **Alun Anderson**

## New UK centre for superconductors

London

BRITISH universities have until 15 September to submit their bids to host the new "University and Inter-Institutional Research Centre" (URC) for high-temperature superconductivity. So says a letter sent to some universities by Professor E. W. J. (Bill) Mitchell, chairman of the Science and Engineering Research Council (SERC), who intends the centre should be allocated by the end of the year.

Although only one centre will be established, there will be collaboration between the host and other universities. The institutions invited to submit are Birmingham, Bristol, Cambridge, Durham, Imperial College London, Leeds, Liverpool, Oxford, Southampton, Strathclyde and Warwick. This centre is the first of several expected to be established soon (see *Nature* 328, 193; 1987).

At a recent meeting, SERC approved £700,000 from a delayed round of grant awards to be spent on superconductor research, and endorsed a £3-million special initiative for the topic, £1 million of which would come from industry and/or the Department of Trade and Industry, plus £1 million each from the Science and Engineering Boards. **Philip Campbell**

## Positive Ariane tests greet motor redesign

Paris

PROBLEMS with the third-stage motor which have grounded Europe's Ariane space rocket for more than a year (see *Nature* 327, 267; 1987) seem at last to have been overcome. Following successful tests by its manufacturers, Société Européenne de Propulsion (SEP), the redesigned motor has now been fitted to the Ariane third stage, ready for shipment to the Kourou launch site in French Guiana. Provided that two further tests, on identical motors, are also positive, a launch date of 11 September was confirmed by Arianespace as "unofficial but realistic".

The final decision of whether to go ahead with the delayed V19 launch will be made at the end of August following further work. Meanwhile, the first two stages of Ariane have been taken out of storage and are being assembled at Kourou. The V19 launch will put the Australian AUSAT K3 and European ECS-4 satellites into orbit and is scheduled to be followed by two further launches this year. With 46 satellite launch contracts on its books, worth FF15,000 million (\$2,470 million), Arianespace needs to carry out 8 or 9 launches a year until 1990 to catch up on its backlog.

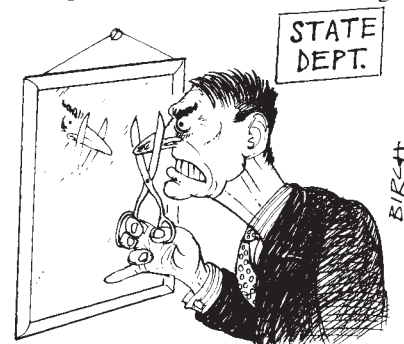
**Peter Coles**

## US doubts over Soviet satellite-launch services

London

SOVIET space experts are disputing a decision of the US State Department to forbid US companies to put commercial satellite payloads into orbit. The State Department says that the ban is to prevent advanced technology from falling into Soviet hands but the Soviet side (particularly the Glavkosmos organization) say the US objection is unfounded.

Glavkosmos, set up in 1985 to provide commercial launch facilities, has been discreetly making its facilities known to those who might otherwise have used US launching systems since the Challenger accident in January, 1986. The Soviet Proton rocket has been advertised as "highly reliable" and the new 2,000-tonne Energiya craft, which can deliver a 100-tonne payload into orbit, is described as a "new generation" device. Launching fees



are "substantially lower" than those of the US National Aeronautics and Space Administration (NASA) or Arianespace — some say about half-price.

The Glavkosmos agency says that it can accommodate the reservations of potential customers. The authorities, it says, will waive customs inspection of satellites, and cautious customers could guard their satellites day and night until they reached their orbits. The agency says that if these concessions do not satisfy the State Department, it can only be that its embargo is political in origin.

The State Department, on the other hand, says that Glavkosmos officials have been "clearly informed" of the "long-standing" US rules that make the use of Soviet facilities by US companies impossible, and says that the Soviet side has also been told that no change of this policy is contemplated in the foreseeable future.

Even so, the backlog of commercial launches built up in the wake of the Challenger accident may offer some hope for Glavkosmos. Between 60 and 70 commercial satellites, valued at \$7,000 million, are believed to be stranded on the ground for lack of launchers. **Vera Rich**



# Stagnant waters flow at last at Sizewell B power station

London

BRITAIN'S nuclear power programme, stagnant for almost a decade, finally resumed its course last week with the start of construction on the Sizewell B pressurized water reactor (PWR) station in Suffolk. Triumphant declaring "the waiting is now over" before clambering on a large crane, Lord Marshall, the chairman of CEBG, inaugurated work on a diaphragm wall, 1,200 m long and 50 m deep. The wall will allow the site to be drained without affecting ecologically important wetlands nearby or the Sizewell A gas-cooled reactor station.

CEGB started looking for a site for its first PWRs in 1977. Preliminary hearings on a planning application were held in 1982, but a full-scale public inquiry was launched in January 1983, and lasted until March 1985.

Layfield's eight-volume report, recommending approval of the project, appeared last January, and the then Secretary of State for Energy, Mr Peter Walker, rubber-stamped the decision in March.

By the time Walker gave the go-ahead, CEBG's bill had reached £200 million. So far, contracts worth £650 million have been placed, with a further 60 contract packages remaining to be awarded, to bring the total cost of the project to £1,600 million at current prices. CEBG intends that the station will be generating 1,175 MW by 1994, and that five or six similar stations across the country will follow, with the first application, for a station at Hinkley Point in Somerset, to be sub-

mitted before the end of this year.

But the PWR programme may need to be revised in view of the government's manifesto pledge to sell the electricity industry to private investors, detailed plans for which are expected by the end of the year. The returns on individual power



*"I am determined that Sizewell B will be built to time and cost". Lord Marshall at the new site.*

stations similar to Sizewell B could be too risky to attract investors. If the CEBG were to be privatized as a single entity, the risks could be spread, but the sale would not give rise to the competition in power generation that many in the government would like to see.

Because CEBG did not expect such a prolonged debate over Sizewell B, no new nuclear power stations had been ordered since 1978. This meant that permission

## Taking science to the Indian masses

New Delhi

TWENTY-SIX People's Science Organisations (PSOs), backed by hundreds of scientists, plan to cover 25,000 km of India by road to share with people the excitement of science and to explain how it can change their lives.

Preparations have already begun for the month-long All-India Science Festival beginning in October. Starting from five different towns, the PSO buses will stop at 500 places. The messages of science will be spread by roadside dramas, songs, films, quizzes, essay competitions and models.

The marathon science march will end on 7 November when 1,000 scientists and 5,000 teachers will join the PSOs in a day-long science 'Woodstock' at Bhopal. Two dozen institutions are associated with the development of exhibits.

Eighty per cent of Indians live in villages where the impact of modern science and technology is not visible. According to its organizers, the festival seeks to "forge active grass-root linkages between scientists and the people at large who are presently alienated from science". The festival should reach 5 million people directly and 50 million indirectly. K.S. Jayaraman

was needed to extend the lifespans of ageing gas-cooled 'Magneox' reactors (the uranium fuel bars are contained in magnesium alloy - 'Magneox' - cans). Of the 26 Magnox reactors built between 1956 and 1971, 11 are still in use.

According to engineering data available at the time of their conception, the reactors were expected to have safe lives of 20-25 years. As a condition of extending the lifespans of the Magnox stations, the government's safety watchdog, the Nuclear Installations Inspectorate (NII), ordered CEBG to carry out a five-year long-term safety review of each reactor. Last week, NII published its assessment of the first review, for the two-reactor station at Bradwell, in Essex, built in 1962 and Britain's oldest nuclear power station.

Although NII accepts that there is no evidence to suggest immediate danger from the station, it lists 17 key requirements that must be met if the station is to continue in operation until 1992. The most serious questions concern weaknesses in the pressure circuits that contain the pressure vessels housing the nuclear core.

CEBG have until October to convince NII that it can meet the requirements, the work for which must be carried out to NII's satisfaction by March 1989. If not, NII will call for the reactor pressure to be lessened, making the station uneconomic to run. CEBG is confident that it can meet the deadlines at a cost of between £3 and £5 million. Simon Hadlington

## Solar reactor to be gamma-ray observatory

Paris

AN experimental French solar energy plant, abandoned since July 1986 because it used more electricity than it produced, is to be turned into a gamma-ray observatory. Based at Thémis in the eastern Pyrenees, this innovative project, called ASGAT (Astronomie Gamma à Thémis), is the result of a collaboration between the Institute for Fundamental Research of the Commissariat à l'Energie Atomique (CEA), which developed the solar plant, and the Institute for Sciences of the Universe (INSU), part of France's leading science research body, the Centre National de la Recherche Scientifique (CNRS). When completed in spring 1988, the observatory will be the largest of its kind.

By using the focusing mechanism and mirror mountings of the Thémis plant, about 60 per cent of construction costs of a new observatory will be saved. The conversion essentially involves replacing the

existing 200 plane mirrors by seven 7-m diameter parabolic mirrors. With the aid of photomultipliers, these mirrors will pick up 'Cerenkov light' produced by gamma-ray photons arriving from deep space, which generate very high-energy particles in the upper atmosphere. The surface area of the Thémis mirrors will be greater than that of all other gamma observatories put together, according to Philippe Goret, the project's director. This will enable the source of the high-energy gamma rays to be located with a precision an order of magnitude greater than possible elsewhere.

The ASGAT project, with a grant of FF2 million (£200,000) from the CEA and INSU, will run for three years initially. Four mirrors will be installed by November 1987 and the remaining three by mid-1988. Goret hopes, eventually, for a Europe-wide collaboration to enlarge the observatory, with double the number of mirrors. Peter Coles

## Reagan AIDS panel disappoints health-care professionals

Washington

PRESIDENT Ronald Reagan last week announced the membership of the commission that will advise him on "a full-fledged strategy for battling AIDS (acquired immune deficiency syndrome)". The president made his announcement during a visit to the National Institutes of Health. Although Reagan referred to the formation of the commission as a "big step against AIDS", doubts immediately surfaced about the qualifications of the panel, raising questions about its effectiveness.

Reagan announced last month that W. Eugene Mayberry, chief executive officer of the Mayo Foundation in Rochester, Minnesota, would chair the 13-member presidential commission. Since then there has been an internal debate at the White House, focusing on the question of whether a homosexual should be included. Before the president's

announcement, word leaked that Frank Lilly, chairman of the genetics department at Albert Einstein Medical Center in New York would be on the panel. Lilly is homosexual.

Lilly is also the only panel member with a graduate science degree. He was a member of the National Academy of Science's committee on a national strategy for AIDS (see *Nature* 324, 3; 1986). The other members with a health background are Woodrow Myers, health commissioner for the state of Indiana, William Walsh, founder of Project Hope, Colleen Conway-Welch, dean of nursing at Vanderbilt University, Theresa Crenshaw, a physician specializing in sexual therapy, and Burton James Lee, a

I have no personal recollection of being told about this AIDS Committee....



physician from New York. John Creedon, chief executive officer of Metropolitan Life Insurance Company, has also been involved in health-care financing.

The commission will make its first report to the president in 90 days, with a final report within one year. The panel's brief is comprehensive. It will make recommendations on research, treatment, ethics and international participation.

But many believe the panel is ill-equipped to cope with the complex questions raised by AIDS. Sheldon Wolff, co-chairman of the National Academy's committee, says he is sorely disappointed with the president's selections.

One of the recommendations of the academy's report was for an independent advisory panel on AIDS. But Paul Volberding of San Francisco General Hospital's AIDS unit — and a member of the academy panel — says the president's panel lacks the strong, well-informed, independent people who could make a difference to the way the country responds to the AIDS problem. **Joseph Palca**

Other panel members are Richard De Vos, co-founder of Amway Corporation, John Cardinal O'Connor, archbishop of New York, Penny Pullen, representative to the Illinois State House of Representatives, Cory SerVaas, editor and publisher of *The Saturday Evening Post*, and Admiral James Watkins, retired.

## Genentech loses another patent dispute in court

Washington

CLOSE on the heels of the British High Court decision ruling its patent for tissue plasminogen activator (TPA) invalid (see *Nature* 328, 189; 1987), Genentech is again the loser. A US judge ruled last week that Genentech is infringing a patent for Factor VIII:C, a natural blood-clotting agent used to treat haemophiliacs, that it is producing by recombinant means.

The patent for Factor VIII:C is held by the Scripps Clinic and Research Foundation of La Jolla, California, and covers the molecule itself and several methods for purifying it from pooled human blood plasma. Scripps licensed the patent to Rorer Group, Inc., which sued Genentech for infringement. Rorer has also sued Chiron and Baxter Travenol, both working on the recombinant factor.

The dispute is seen as the first trial case of "an isolator versus a cloner". Several of the major products being developed by biotechnology companies using recombinant DNA technology may also be isolated from blood or tissue. Although things found in nature cannot be patented, current US patent law provides protection for companies that find a way to produce a substance in a form that is "more pure" than that which occurs naturally. Factor VIII:C is the active clotting portion of the Factor VIII complex found in blood, and purifying it is therefore patentable.

The purity of Factor VIII is a central issue, because the handful of current producers get their plasma from paid donation centres. Until 1985, donors were not screened for the AIDS (acquired immune deficiency syndrome) virus, even though a significant proportion of them had used intravenous drugs. As a result, according to a National Haemophilia Foundation study, two-thirds of the roughly 20,000 haemophiliacs in the United States have been infected.

Although donor-screening and heat-sterilization are now routine for Factor VIII manufacturers, producing it by recombinant DNA techniques would totally remove the risk of viral infection. Accordingly, the judge did not issue a preliminary injunction to prevent Genentech from making or using recombinant Factor VIII:C, as "haemophiliacs could suffer if there is any delay in bringing recombinant [Factor VIII] to the market".

Rorer offered to license Factor VIII:C to Genentech, but Ernest Lipscomb, Rorer's patent counsel, said Genentech told them in effect that they could go to Hell. Genentech plans to appeal against the decision. **Carol Ezzell**

## Barry Jones bounces back into new post

Sydney

THE reshuffle following the Australian Labor party's election victory will leave the former Minister for Science, Barry Jones, with considerable responsibility for science, despite the disappearance of his own department. In the new government he has been given a ministerial post assisting the Minister for Industry, Science and Commerce, Senator John Button.

The appointment will allow him to continue the restructuring of the Commonwealth Science and Industrial Research Organization (CSIRO) and to expand the Commission for the Future, a pet project he set up in 1984. In the Department of Industry, Technology and Commerce (DITAC), the commission may be able to take on a function similar to that of the US Office of Technology Assessment.

Other changes that have reunited responsibility for science and technology in one ministry have been welcomed. In particular, the transfer of the Australian Nuclear Science and Energy Organisation from the Ministry of Minerals and Energy to DITAC should bring an end to territorial squabbles with CSIRO. Doubts still remain over the promised creation of the Australian Research Council. The government seems to be thinking of putting it in with the Department of Education, Employment and Training rather than DITAC even though the report that recommended its establishment argued that it should be answerable to the Minister for Science. **Charles Morgan**



# High-finance approach to protecting tropical forests

Washington

In one stroke, Bolivia last week reduced its external debt by \$650,000 and protected 3.7 million acres of tropical forest. The twin achievements came as part of a deal with Conservation International, an environmental organization, which purchased the Bolivian debt in exchange for the land protection agreement. Bolivia also agreed to endow a fund worth \$250,000 in local currency to pay for management of the newly protected land.

Countries with large external debts have sought to increase exports as a way to obtain cash for making loan payments,

in exchange for establishing conservation easements. The bill also requires the US Secretary of the Treasury to conduct a study of the status of tropical forests. According to Porter's staff, the World Bank has been enthusiastic about the bill, and Porter is optimistic about its chances of being passed. Similar legislation is being introduced in the Senate.

The heavy discounting of debts from developing countries has opened another channel. Barbara Bramble of the National Wildlife Federation says some country's debts are discounted by 90 per cent. She is promoting legislation to allow banks to

write off their loans, claiming a tax reduction for the whole loan rather than the market value. In exchange, the debtor countries would take steps to protect their natural resources. Bramble admits, however, that this scheme will go only a little way towards reducing the external debt of more than \$1 million million of developing nations.

Some countries have taken their own steps. On 25 July, Costa Rica will open the Guanacaste National Park, located on the Santa Elena peninsula in the northwest corner of the country. The park will include 32,500 acres obtained as part of a programme run by the Costa Rican National Park Foundation to purchase Costa Rican debt in exchange for enlarging and managing national parklands.

Joseph Palca



often by clearing forests for cash crops and additional agricultural land. But conservation groups have been trying to prevent countries from exploiting irreplaceable natural resources. The 'debt-for-nature' exchange appeals to many as a solution.

In the Bolivian example, Conservation International bought the \$650,000 note at an 85 per cent discount using a \$100,000 grant from the Frank Wenden Foundation. The foundation is giving \$100,000 to the World Wildlife Fund to make a similar arrangement with Costa Rica.

The newly protected region in Bolivia surrounds the Beni Biosphere Reserve, a 334,200-acre tract established in 1982. The reserve supports 13 of Bolivia's 18 endangered species. The new reserve is protected by congressional law, as opposed to the administrative decrees that formerly covered the Beni Reserve.

Other approaches are being pursued in the US Congress. Last week, Representative John Porter (Republican, Illinois) introduced the Tropical Forest Protection Act of 1987. The bill would initiate two three-year World Bank pilot programmes to discourage damage to forests. In one programme, loans would be made to induce sustainable use of tropical forests, such as rubber-tapping or nut-farming. The other would suspend debt payments

## Sarawak's tropical rainforests exploited by Japan

Tokyo

JAPAN has used overseas aid to help finance the exploitation of Sarawak's tropical rainforests. Evelyn Hong, a Malaysian anthropologist, revealed last week that the Japanese International Cooperation Agency (JICA) provided a ¥200 million (\$1.3 million) soft loan in 1982 to build a logging road and bridge in the Limbang district of Sarawak where more than 70 per cent of the forests have been earmarked for logging, largely for export to Japan.

Hong visited Japan to publicize the plight of the Penan, a tribe of 5,000–6,000 nomadic hunters who live in the logging area and depend on the forest for their livelihood. During the period 1963–85, 2.8 million hectares, or 30 per cent of Sarawak's total forest area, were logged and in 1984 a further 5.8 million hectares were licensed out. In a last-ditch effort to try to stop the wholesale destruction of their forest, the Penan are manning barricades across several of the logging roads, including that built with Japanese aid.

More than 80 per cent of Japan's hardwood imports come from Malaysia, about 40 per cent from Sarawak. Much of the imported wood is used for disposable panelling for forming concrete during construction work. Japan's planned construction projects, such as the Tokyo Bay bridge, may lead to increased hardwood imports and more rapid depletion of Sarawak's forests. Imports from Sarawak have risen dramatically recently partly because other countries such as Indonesia and the Philippines have clamped down on the export of unprocessed logs — Japan levies import tariffs on processed timber and most imports are raw logs.

Most of the logging concessions in Sarawak, valued at thousands of millions of

dollars, are owned by close relatives of leading state politicians, and the Penan have had to go to the federal government in Kuala Lumpur to appeal their case.

Hong is concerned that Japan's recent promises drastically to increase overseas development aid may result in more projects of dubious value to local inhabitants. The Limbang logging road was built with a 10-year loan from JICA; Foreign Ministry officials admit that no environmental impact study was carried out before or after road construction. The Penan claim that the logging operations have destroyed their traditional hunting grounds and that run-off from the logged areas has led to pollution and silting of the rivers.

The construction of the Batang Ai Dam in Sarawak, which destroyed more than 20,000 acres of forest and caused the displacement of thousands of natives, was financed by, among others, the Asian Development Bank (to which Japan is the major contributor), the Overseas Economic Corporation Fund of Japan and Mitsui Trust and Banking Company. The chief contractors were Maeda and Okumura construction companies of Japan.

The Environment Agency (of Japan) is considering legislation for the environmental impact assessment of overseas development projects but is not expected to take action for several years. The International Tropical Timber Organization has set up headquarters in Yokohama with a mandate to protect tropical rainforests but has been struggling to start operations on limited funds (see *Nature* 328, 537; 1987). Meanwhile the Penan, who set up their first blockade in March and are now manning twelve, have vowed to block the roads "until they die".

David Swinbanks



# Boycott of South Africa

SIR—John Maddox's article "Science in Apartheid" (*Nature* 327, 269–276; 1987) is essential reading for anyone wanting to come to a conclusion about the correctness of the boycott tactic. However, the tone of your leader "Boycott someone and feel virtuous", and especially its sub-heading "The academic community should find a better way of making its good intentions come true" make me realize that a number of ambiguities remain in the Maddox article, ambiguities which led you to a conclusion opposite to that to which most of us would have come.

The major problem seems to lie in Maddox's statement that "one person, one vote, tomorrow would indeed be a recipe for disaster". The ambiguity here is: disaster for whom? Certainly for the privileged whites — but equally for the inhabitants of Soweto? Has, as Maddox writes, the non-white majority no political structure within which to organize its opinion? Why then is the African National Congress so courted by the great industrialists and now by the Progressive Party? The transition to a non-racial democratic society will surely be hard, but it must start today, not tomorrow, and with "one person, one vote". This ambiguity leads him to his second difficulty: what would be the conditions in which the boycotters would relent? If one is clear that "one person, one vote" is the blueprint for a South Africa acceptable to the black community, then the attainment of universal franchise is the point at which the boycott ceases.

We should be unambiguous about the aim of an academic boycott. Rather than to allow us to feel virtuous, its aim is to weaken the apartheid state so that its rulers become unable to hold power and proceed, rather, to negotiate with the African National Congress on the transfer of power. We should be clear, too, that this boycott is free of the liberal's major hesitation about economic sanctions. The latter obviously hurt blacks. Should they be asked to suffer if their voice in this matter is not given a hearing (as it cannot be tested by the ballot box)? As far as a science boycott is concerned, if South African science ceases for the five or ten years that might lie ahead before majority rule, hardly a black will suffer. Few are employed by the scientific establishment, little of the immediate results of this science will affect their standard of living. Those who will feel the effects, of course, are the scientists themselves, almost all of whom are white and privileged. But that is a function of the boycott, to make these scientists understand that their working in the South African scientific community is at least passive support for the apartheid system.

An issue that is still not clear to me is whether the boycott should be total. Perhaps the academic boycott needs its Sullivan Code. An international body could set up criteria for enabling journals to accept papers from South African authors who state their categorical rejection of apartheid, and who are from institutions that have publicly dissociated themselves from that system. Public discussion on these issues in South Africa will itself have important educational effects on the scientific community and beyond it. And the organizers of scientific meetings might consider what was discussed among the sportsmen and welcome, at international meetings, 'mixed' scientific delegations from South Africa. This stipulation would itself greatly encourage the acceptance of blacks into the graduate schools of the South African universities and their more rapid advancement to faculty positions. Scientists at institutions that refuse to accept the new code should be offered facilities abroad until they can return to a, hopefully, non-racial South Africa (Azania?) when this is set up.

It is important that the West seriously confronts the human problem of the post-apartheid South Africa, which will involve the resettling of those people of all races who will feel unable to remain in South Africa under a new regime. Rather than making it difficult for South Africans to emigrate to the United States, Canada and the United Kingdom (and the Netherlands and France), they should already at this stage be encouraged to do so, and assisted in their resettlement.

W.D. STEIN

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SIR—Your feature on "Science in Apartheid" (*Nature* 327, 269–276; 1987) admirably challenged the smug ivory tower position that supposes science and scientists can be neutral and ignore the boycott issues.

No longer is the South African Council for Scientific and Industrial Research (CSIR) able to recruit 100 foreign scientists and technologists each year; no longer can South Africa sustain its science-intensive economy through overseas recruitment. Moreover, you make it clear that we now have a good chance through argumentation and ostracism of reversing the skill drain to South Africa and so hurting the CSIR and other government bodies.

**Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.** □

The CSIR differs of course from the British Science and Engineering Research Council (SERC). Not only does the CSIR cater for military as well as civil research, but it is also engaged in economically important areas such as nuclear technology, oil-from-coal and mineral extraction/processing.

One objection to the SERC link with the South African Astronomical Observatory (SAAO) was that CSIR personnel attended the UK Conferences on Southern Hemisphere Astronomy — people who were not astronomers but who could have been scouting for technological developments (satellite tracking, remote sensing, image-processing software) and for scientific specialists. Mysterious South African names similarly appear on the attendance lists of big international conferences such as that of the International Union for Geodesy and Geophysics.

The boycott clearly does harm science, in the short term. A programme to observe Halley's comet in which I was involved, having been turned down for the Anglo-Australian Telescope and diverted by SERC to the SAAO, instead made use of a significantly less suitable Californian telescope. On the other hand, apartheid is harming science and depriving potential scientists just because of skin colour.

Individual scientists lose out, unfortunately, but so do the South African blacks, who are deprived of higher education, let alone the chance of becoming scientists. We should now be confident that the scientific boycott can help to hasten the downfall of apartheid, and in the longer term benefit the cause of science as well as humanity.

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## Brave new world

SIR—The Commentary by Erwin Chargaff, "Engineering a molecular nightmare" (*Nature* 327, 199; 1987), not only shows Chargaff's wit and wisdom, but also his moral courage to stand up against the unacceptable. I disagree only with his assertion that "a gigantic slaughterhouse, a molecular Auschwitz, in which valuable enzymes, hormones and so on will be extracted instead of gold teeth" is to come. I rather think it has already arrived in the massive industrial exploitation of aborted fetuses, from which "beauty products" are made.

FERNANDO ORREGO

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# Human scale high-energy physics

*Apart from the occasional look backwards, it is almost forgotten that the foundations of high-energy physics were laid not by the design of experiments but by the observation of chance phenomena.*

NOSTALGICALLY, the University of Bristol was last week celebrating the fortieth anniversary of two developments in what is now called high-energy physics which, in retrospect, mark the watershed between the heroic past and the machine-based present. An understanding of how matter is constructed has been an obvious prize since Aristotle's time. Until 1947, progress resulted from people working in ordinary laboratories gleaming what they could, by ingenious design of equipment and sheer hard work, from the chance occurrence of cosmic-ray events.

But already, in 1947, it was clear that the future would lie with the accelerators then being built. That human-scale techniques should have produced in a single year the surprises that then emerged, justifies last weeks' celebrations; that those developments should have turned out to be the last important contributions of British experimentalists to the field may also have been in people's minds.

Historically, 1947 was a chaotic year. Much of the British physics community was occupied with the foundation of the Atomic Energy Research Establishment at Harwell, then a muddy disused airfield, to which groups of academics and their students were repeatedly conducted in the hope that they would stay. (Many did.)

Particle physics, by contrast, was simple. There were protons and neutrons (united in the single entity called a nucleon by the concept of isotopic spin), electrons and positrons (related by Dirac's theory of the latter as holes in a sea of electrons of negative energy), neutrinos (postulated on theoretical grounds by Pauli and Fermi, but not observed) and 'mesotrons' — the particles postulated in 1935 by Yukawa as the mediators of the forces between nucleons, recognized (by their mass, intermediate between that of an electron and proton) among the particles of the cosmic rays and named by C.D. Anderson and S.H. Neddermeyer (*Nature* **142**, 878; 1938) on the basis of observations at the California Institute of Technology. This sparse list of 'fundamental' particles survived the Second World War unchanged.

On reflection, it is odd that mesotrons had been as readily welcomed as Yukawa's hypothetical particles. True, the mass was, as expected, intermediate between that of the electron and the proton (although the difficulty of producing exact estimates suggested to some

that mesotrons might not be unique). They were most easily recognized in the component of the cosmic-ray stream penetrating substantial amounts of matter, behaving as if they were 'heavy electrons' (their familiar name) rather than as the mediators of nuclear forces (which should interact strongly with nuclei).

The first development of 1947 was C.F. Powell's success at Bristol in making sense of mesotrons (Lattes, C.M.G., Muirhead, H., Occhialini, G.P.S. and Powell, C.F., *Nature* **159**, 694; 1947). Powell's contribution had been to work away, for the best part of a decade, at the use of photographic emulsions for extracting usable information about cosmic-ray particles from the tracks of exposed silver grains scattered along the paths of quickly-moving charged particles. As the world knows, it was first necessary to persuade manufacturers to make emulsions thick enough to be useful, then to develop the techniques for observing tracks a few  $\mu\text{m}$  long and then to work out the rules by which the momentum of a particle could be inferred from the density of exposed grains along a track and from the angles through which it appeared microscopically to have been scattered. The upshot was the great surprise that there are, indeed, two kinds of 'mesotrons' — muons and pions, the 'heavy electrons' and the Yukawa particles respectively. The masses, as now measured, are the equivalent of 106 and 140 MeV respectively, different enough to account for the indecisions of the 1930s.

The menagerie of fundamental particles was quickly extended with the publication, later that year, of another unexpected cosmic-ray observation — two oddly shaped tracks in separate cosmic-ray cloud chamber photographs taken at the University of Manchester by G.D. Rochester and C.C. Butler (*Nature* **160**, 855; 1947). One of the two photographs was of a pair of tracks, pointing downwards beneath a lead plate in the chamber, in the V-shaped configuration. The second, even more surprising, shows a particle with minimum ionization suddenly bent through an angle of 19 degrees — and then traversing several centimetres of lead while deviating by only 3 degrees from its original direction.

The two particles were named V and K particles. P.M.S. Blackett, the head of the Manchester physics department, energetically arranged that anybody who could

operate a cloud chamber in a magnetic field should be sent off, with the appropriate equipment, to look for further examples of the same phenomena, for which the name 'strange' was coined because of their surprisingly long lifetime against spontaneous decay. The Pic du Midi, France and the Jungfrau Joch, Switzerland were usual destinations.

There was a famous occasion when Blackett, then also the chairman of the stripling National Research Development Corporation, invited his august committee members to join him on the night train from London to Manchester to inspect the equipment with which this great discovery had been made. Blackett, still very much the naval officer, is believed to have uttered words more appropriate to the forecandle than to the quarter-deck when he found that hapless Rochester had dismantled his cloud chamber for cleaning.

What the cloud-chambers had found was the first evidence of the quantum number now fittingly called strangeness, an attribute of a quark (the notion of which had not then been invented). The cosmic-ray people were cock-a-hoop that so much had been found so quickly. Must not these testimonials to their ingenious craft keep the accelerators at bay?

That was hoping against hope. The accelerators had logic on their side. People began drifting off into other fields. Manchester's head technician, one Thomas Ball, a man with green fingers for building cloud chambers, became the head technician at CERN at Geneva and spent the rest of his working life negotiating contracts with the Brown Boverie's of this world. It is not that cosmic-ray physics died, but that it became part of observational astrophysics (while the already elaborate counter-controlled cloud chambers became the precursors of the detectors that now dance attendance on the accelerators).

Even so, some may note, it needed the passage of a full quarter of a century before the next quark attribute (charm) was identified by accelerators at Brookhaven and Stanford. What was lost was the fun of seeing how people's ingenuity and dexterity can be made to breed discovery. It is wry that all this was apparent at the point at which cosmic-ray physics had yielded its most spectacular results, which is one reason why Bristol's celebration last week must also have been something of a wake. **John Maddox**



## Superconducting ceramics

# High-temperature anomalies

David Caplin

Two strands are emerging in the helter-skelter race to find higher transition temperatures for superconductivity. One is the application of textbook scientific method in the communal verification of extraordinary results. Once the scientific community woke up to the implications of J.G. Bednorz and K.A. Muller's report (*Z. Phys.* **B64**, 189–193; 1986) of superconductivity above 30 K in  $(\text{La, Sr})_2\text{CuO}_x$  compounds, anyone who wished to could reproduce the results within a couple of days. There is the story, perhaps apocryphal, that having received the manuscript announcing the discovery of the  $\text{YBa}_2\text{Cu}_3\text{O}_x$  90-K superconductor (Wu, M.K. *et al. Phys. Rev. Lett.* **58**, 908–911; 1987) in the morning postal delivery, one of the referees had confirmed the transition temperature before midnight.

On the other hand, there are now at least a dozen reports, including one from Huang *et al.* on page 403 of this issue, of anomalies that indicate superconductivity in the same materials at far higher temperatures, but even the authors themselves are unable to reproduce their results from one sample to another. Often, the electrical resistivity appears to drop by several orders of magnitude, or the magnetic susceptibility suddenly becomes strongly diamagnetic.

It is not that careful scientists find the reproducible data and the careless find the anomalies, for several groups have produced both species. The common feature of reports of high-temperature anomalies, at 155 K (Ovshinsky, S.R. *et al. Phys. Rev. Lett.* **58**, 2579–2581; 1987), at 'room temperature' (Sumitomo Electric Co; see *Nature* **328**, 98; 1987) and at 230 K (Huang *et al.* on page 403), appears to be that the superconducting phase lives for a few hours or days at most, and that it constitutes only a small proportion of the total volume.

An important point, well known in conventional superconductivity, is that a minute amount of superconducting phase can provide zero-resistance percolative paths in a non-superconducting matrix. Grain boundaries, to which minority phases tend to segregate anyway, are particularly favourable locations. Magnetic tests for superconductivity can be equally misleading, because any closed path of zero resistance will exclude flux changes from its interior, and so give a large diamagnetic signal. (This is often described, misleadingly and incorrectly, as an observation of the Meissner effect; the latter is the *expulsion* of magnetic flux from a superconductor as it is cooled, in a

magnetic field, through its superconducting transition.)

In many cases the samples showing the anomaly had been prepared by a slight variation on the known optimum recipe for the  $\text{YBa}_2\text{Cu}_3\text{O}_x$  compound, for example by replacing some oxide with fluoride (Ovshinsky *et al.*) or by exposing the material to argon at 300 K (Huang *et al.*), which would presumably cause the sample to lose a small amount of oxygen.

This circumstantial evidence suggests that the material that is responsible for the high-temperature effects is not a true bulk phase, in which case endeavours to isolate it are doomed to failure (but who knows what may have happened by the time these comments appear in print). It could be instead that in special circumstances, there is some kind of two-dimensional superconductivity at grain boundaries. This would not be inconsistent with what is known already about the bulk high-temperature superconductors: the active regions in these perovskite-related structures are the Cu–O planes, and the superconducting properties are extremely sensitive to occupancy of the oxygen sites.

Oxygen diffusion rates in these compounds are high (they are well known to be superionic conductors at 400°C and above), but are far from being high enough to allow significant structural

change in bulk material at room temperature. The ceramic superconductors as presently prepared are porous, however, and the diffusion rate for oxygen along grain boundaries could well be orders of magnitude higher than the bulk value. Thus, it is not unreasonable that during hours or days at room temperature, the oxygen stoichiometry of a superconducting intergranular region could change significantly.

The irreproducible effects that are being seen in the superconducting oxides resemble the anomalies reported 10 years ago (Brandt, N.B. *et al. JETP Lett.* **27**, 33–38; 1978) in  $\text{CuCl}$  under pressure. These were then investigated in considerable detail (Chu, C.W. *et al. Phys. Rev. B* **18**, 2116–2123; 1978), but very little in the way of systematic behaviour could be identified. Various mechanisms were considered, including superconductivity induced at an interface by an excitonic mechanism, but without any firm conclusion.

Reports of possible, if transient, superconductivity at these higher temperatures should not be brushed aside. It is vital to discover a reliable way of preparing the material that displays these anomalies; there will then be much hard work to understand their origin. It may be that to create room-temperature superconductors we have to replicate on a macroscopic scale the microscopic structure of grain boundaries, perhaps using the techniques of molecular-beam epitaxy. □

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## Quasars

# Probing the early Universe

R.F. Carswell

For those interested in the history and the ultimate fate of the Universe, quasars have been a source both of hope and of frustration. Hope because quasars are so bright, and seen at such large distances, that in the highest-redshift cases we see them as they were when the Universe was at most one-fifth of its present age. Thus the study of quasars should allow us to infer some of the properties of the early Universe, and particularly any changes in the rate of expansion since then. Frustration because quasars show such diverse behaviour, most importantly in their intrinsic luminosities, that any effects caused by the expansion of the Universe are completely masked. The prospect of disentangling the evolution of quasars from changes in the expansion of the Universe is remote today. But there is another use for quasars: to probe gas clouds that intervene between us and them. This was

the subject of a recently held workshop\*.

The spectra of all high-redshift quasars show atomic-absorption lines arising in gas clouds with redshifts, and hence distances and ages, up to that of the quasar itself. Mostly we detect only the lines of neutral hydrogen, the most abundant element in the Universe. In a few cases, though, heavy elements are found, indicating some nuclear processing in stars. Recently, high-quality, high-resolution spectroscopy has enabled astronomers to address detailed problems concerning the nature of these quasar-absorbing clouds.

Detailed work by A.M. Wolfe (Pittsburgh Univ.) and his collaborators shows that if the regions that contain heavy elements are in galaxies, then the galaxies must have been considerably larger in the past than they appear to be now. This

\*QSO Absorption Lines: Probing the Universe Space Telescope Science Institute, Baltimore, 10–21 May 1987.

raised questions that were addressed by various speakers. R. Sancisi (Groningen Univ.) said that some of the nearby galaxies that he had studied using the 21-cm-wavelength (neutral-hydrogen) line are very extended, so even nearby galaxies may be larger on average than has been believed. But there is considerable uncertainty in comparing 21-cm radio data with optical (or ultraviolet) data, mainly because the radio technique can detect neutral hydrogen only at densities 10,000 times larger than those routinely detected optically, simply because of the difference in line-strength. F. Briggs (Pittsburgh Univ.) showed how the detailed velocity structure and the spatial extent of the strongest hydrogen absorbers can be inferred using 21-cm absorption against a quasar.

Even in the early Universe, the abundances of heavy elements were not very different from those found in the interstellar medium in our Galaxy today, which indicates that early nucleosynthesis and enrichment of the interstellar medium by massive stars probably occurred (J. Bergeron, Univ. Paris; C. Blades, Space Telescope Science Institute). The systems seen all have similar velocity structures as might be expected if they are in galaxies (W. Sargent, Caltech). A major difference between the high-redshift material and the galactic interstellar medium is found in the  $H_2/H$  ratio, which has a value near unity in many regions in our Galaxy. Quasar 0528-250, one of two which were investigated, reveals a system with redshift  $z \sim 2.8$  in which  $H_2/H$  is about  $10^{-1}$ .

Another link with real galaxies was indicated by B. Savage (Wisconsin Univ.) who described the ultraviolet observations of the interstellar medium in our Galaxy. In particular, the key tracers for the hot interstellar medium are highly ionized species such as  $N^{++}$  and  $O^{++}$ . These are rarely seen in quasar absorption systems, but are common in our Galaxy. This might be a genuine difference, but the situation is often ambiguous. The  $N^{++}$  and  $O^{++}$  lines in the quasar spectrum could be swamped by neutral-hydrogen (Lyman- $\alpha$ ) lines with lower redshift than the quasar, and so they could have been missed. In a further comparison with galactic interstellar medium, D. York (Chicago Univ.) highlighted the difficulty of finding low-redshift systems similar to the high-redshift absorbers, and suggested that gas-rich systems such as galaxies undergoing bursts of star formation could be appropriate candidates.

The hydrogen lines themselves were also discussed. Although an individual quasar spectrum will generally reveal only a few systems containing heavy elements, there are invariably many — several hundred in the highest-redshift cases — absorbing regions in which only hydrogen is

## A case of mistaken identity in swordtail fish

WHY do members of different species avoid mating with each other? The classical explanation is that hybrid inviability results in selection for premating isolating mechanisms<sup>1</sup>. As we have discussed<sup>2</sup> in a previous News and Views article, an alternative route is sexual selection: mating preferences have an evolutionary dynamics of their own, and can evolve in the absence of contact with other populations or species<sup>3,4</sup>. If a geographical barrier divides a population into two, mating preferences can evolve to differ in the subpopulations. When the barrier is removed, choice of

that this preference is a consequence of the elaborate courtship display of the male *X. nigrensis*. Unfortunately, the exact basis of the *X. nigrensis* female mating preference remains unknown because Ryan and Wagner did not perform the same analysis using these females.

What do these results mean in evolutionary terms? The females of the two species have very similar mating preferences, but *X. nigrensis* males seem to have responded much more strongly to this sexual selection than *X. pigmaeus* males. This difference between the species suggests

that the female preference has not been produced entirely by coevolution with the preferred male character, as is implied in many models of sexual selection<sup>3,6,7</sup>. The mating preference may instead have been present in the common ancestral species, and the male *X. pigmaeus* fail to respond either because they lack the necessary genetic variation or because they are

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The larger fish is the swordtail *X. nigrensis*, the smaller is *X. pigmaeus*. (Courtesy of Michael J. Ryan.)

mate alone could prevent hybridization between the two populations. However, a recent study by Ryan and Wagner<sup>5</sup> of swordtail fish suggests that hybridization between populations can sometimes be favoured by sexual selection: females of one 'species' actually prefer to mate with males of another.

In the swordtail species *Xiphophorus nigrensis*, only the larger males have an elaborate courtship display and it is those males that are preferred as mates by conspecific females. Sexual selection therefore favours males with some combination of large body size, elaborate courtship, and perhaps a further, undetected character with which both of these are correlated. *X. pigmaeus* males lack the swordtail that is found in all *X. nigrensis* males, but are like small *X. nigrensis* males in both size and courtship behaviour (see figure). Given a visual choice, *X. pigmaeus* females approach all but the smallest *X. nigrensis* males in preference to those of their own species. Subsequent tests demonstrated

subject to some opposing selective force (such as high predation rates) on courting males. Sexual selection may thus have made hybridization between the two species more likely if their ranges come to overlap. The mating preference of the *X. pigmaeus* females for most classes of *X. nigrensis* males would result in an excess of this type of hybrid mating. Because matings between the species appear to produce viable, fertile offspring, the two species could become one.

Linda Partridge &  
Paul H. Harvey

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detected. Several investigations into the dependence of the number of these latter systems on redshift conclude that they were more numerous in the past. Detailed studies, mainly by R.W. Hunstead (Sydney Univ.) and his collaborators, and by D. Tytler (Columbia Univ.) show that the numbers of detectable clouds decrease as their redshift becomes close to that of the quasar. This is probably because proximity to the bright quasar causes more of the hydrogen to be ionized and, because we can detect only the neutral hydrogen, fewer systems are seen. By extension, J.P. Ostriker (Princeton Univ.) pointed out

that if there was a quasar between us and the quasar against which the absorption is seen this would also reduce the numbers of hydrogen lines observed. This possibility remains to be tested.

Another critical quantity of these so-called 'Lyman forest' systems is the velocity spread revealed by the lines. Published estimates suggest that the full-width-half-intensity for the velocity distribution is typically 50-60 km s<sup>-1</sup>. If this width is caused solely by thermal motion, then it implies temperatures of about 70,000 K, considerably higher than theories suggest. The most likely explanation



is that the lines, although resolved, are not revealing their true component structure given the quality of the available data. More recent results from Hunstead and his collaborators suggest that this may well be the case, but the situation is unclear because of potential difficulties in analysis.

The general view is that the hydrogen clouds are likely to be mainly intergalactic, and pressure-confined by a hot intergalactic medium. As M.J. Rees (Cambridge Univ.) pointed out, however, there are alternative explanations that do not require a hot intergalactic medium, thus avoiding the problem of the origin of the heat. These models generally require the clouds to be bound gravitationally as would happen if they were associated with cold dark matter.

Quasar absorption lines can also probe the environment of the objects. Foltz showed that for radio-loud quasars there is a significant excess of absorbing material near the quasar redshift, and such material seems to be rare in radio-quiet quasars. Cause and effect have yet to be established in this case. By contrast, spectra with very broad absorption lines, arising from material ejected at very high

speeds from the quasar, are usually radio quiet. D. Turnshek (Space Telescope Science Institute) finds that there are some anomalies in the emission line characteristics of the quasars giving rise to the mass outflow. These may be explained in terms of reprocessing of the quasar radiation by the absorbing gas.

As with the quasars themselves, quasar absorption lines have not provided the insights into the nature of the Universe that might be expected. They are, however, superb probes of the earlier stages of galaxies. The fact that we are able to make reasonably detailed comparisons with expectations based on our local knowledge is a sign that the subject has reached a new level of maturity. It is also true to say that we have accumulated sufficient data for some of the old controversies to be buried. And now hypotheses as to the origin and nature of the clouds are described as either 'consistent with' or 'inconsistent with' the observations. What remains now is to reduce the number of 'consistent' versions. □

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## Cellular biochemistry

# Proteins as molecular chaperones

John Ellis

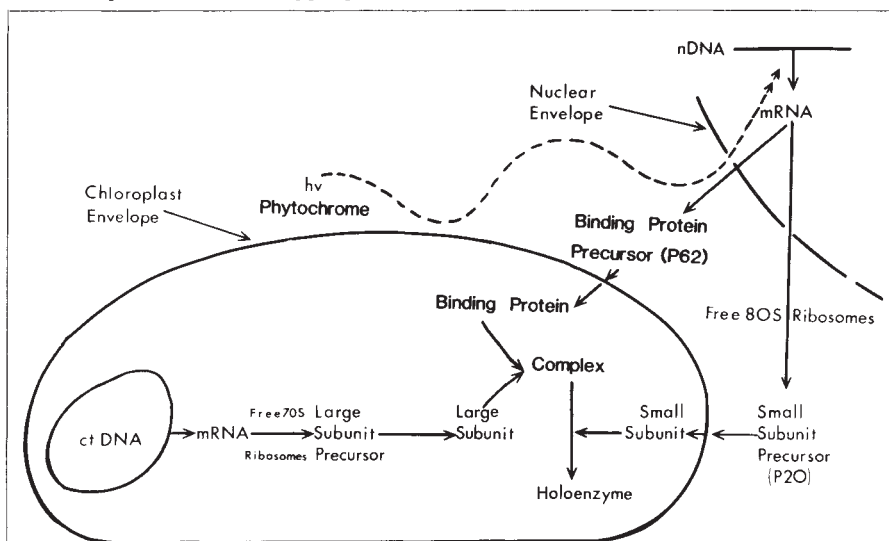
At a recent meeting\* I proposed the term 'molecular chaperones' to describe a class of cellular proteins whose function is to ensure that the folding of certain other polypeptide chains and their assembly into oligomeric structures occur correctly. The existence of such a class of proteins was suggested last year<sup>1</sup> in a speculative article by Hugh Pelham. The basic argument is that in some cases the transient exposure of hydrophobic or charged surfaces normally involved in domain interactions, either within or between polypeptide chains, can result in 'improper' interactions to produce incorrect structures. Molecular chaperones would prevent the formation of such incorrect structures. Pelham also suggests<sup>1</sup> that these proteins can disassemble aggregated structures either that are no longer required or that form during stresses such as heat shock.

The term 'molecular chaperone' was first used by Laskey *et al.*<sup>2</sup> to describe nucleoplasmin, an acidic nuclear protein required for the assembly of nucleosomes from DNA and histones in extracts of eggs of the toad *Xenopus*. Rapid mixing of separated DNA and histones at physiological ionic strength results in a precipi-

tate, but if nucleoplasmin is present, nucleosome cores form<sup>2-4</sup>. Nucleoplasmin does not bind to DNA or to nucleosomes, but interacts with histones in such a way that it shields their positive charges. The term 'chaperone' is thus appropriate as

nucleoplasmin both promotes histone-histone interactions by reducing electrostatic repulsion between them, and minimizes the formation of insoluble non-specific aggregates by competing with the electrostatic attraction between the histones and DNA<sup>4</sup>. Nucleoplasmin is required only for assembly and does not itself form part of the nucleosomes, nor does it carry any steric information specific for nucleosome assembly — this information resides in the histones<sup>4</sup>. It is part of the proposed more general definition of molecular chaperones that they do not form part of the final structure nor do they necessarily possess steric information specifying assembly.

Proteins that fall within the proposed class of molecular chaperones have been described from viral, animal and plant sources. The main constituent of the capsid of bacteriophage T4 cannot produce an organized structure by self-assembly. The main protein of the capsid, p23, aggregates randomly into lumps at the plasma membrane unless another protein, p22, is also present. The p22 forms a core around which p23 assembles into the prohead; the core is subsequently cleaved into small fragments<sup>5</sup>. The lumen of the endoplasmic reticulum of lymphoid cells contains a protein which binds non-covalently to newly synthesized immunoglobulin heavy chains; this protein is called the immunoglobulin heavy-chain binding protein or BiP<sup>6,7</sup>. BiP binds to heavy chains that do not have light chains attached, and seems to recognize the hydrophobic region of the heavy chain that is subsequently covered by light chains. Addition of ATP *in vitro* causes the release of BiP from heavy chains, and the role of BiP in antibody-secreting cells could be to prevent or reverse the



Proposed role of molecular chaperone in the assembly of Rubisco in higher plants. Rubisco large subunits are synthesized from chloroplast genes (ct DNA) whereas Rubisco small subunits and the binding-protein subunits are synthesized from nuclear genes (nDNA). (Modified from ref. 14.)

\* NATO Advanced Study Institute Plant Molecular Biology 1987 Carlsberg Laboratory, Copenhagen, 10-19 June 1987.

formation of heavy-chain aggregates and thus aid the assembly of immunoglobulin molecules<sup>8</sup>.

Note that this heavy-chain binding protein occurs in many cell types and thus may be involved in the solubilization and assembly of proteins other than immunoglobulins. For example, fibroblasts starved of glucose produce in the endoplasmic reticulum a protein termed grp 78 which is identical to BiP<sup>9</sup>; grp 78 could bind to abnormal underglycosylated proteins that accumulate in such fibroblasts and prevent them forming insoluble aggregates<sup>1</sup>.

Part of the traditional duties of the human chaperone is to disrupt 'improper' interactions that might have occurred despite her vigilance. Other candidates for molecular chaperones are heat-shock and related proteins that occur in unstressed cells. Many nuclear proteins become insoluble on heat-shock of mammalian cells, especially preribosomes. The heat-shock proteins hsp 70 and hsc 70 migrate to the nucleolus of heat-shocked cells and bind to these preribosomes. The binding of hsp 70 to heat-shocked nucleoli can be reversed *in vitro* by ATP, leading to the notion that cycles of binding and release may promote disaggregation of insoluble aggregates<sup>9</sup>. There is no direct evidence for this suggestion, but the recovery of normal nucleolar morphology occurs much more rapidly in cells that have been pre-loaded with hsp 70 by transfection with a plasmid that expresses the gene encoding hsp 70 constitutively.

Animal cells also contain proteins related to hsp 70 that are synthesized in the absence of stress and remain in the cytoplasm; hsc 70, for example, binds to coated vesicles in the presence of ATP and causes the disruption of clathrin-clathrin interactions. Thus, a general property of the hsp 70 family of proteins may be the ability to disrupt protein-protein interactions. Moreover, hsp 70 is approximately 60 per cent homologous at the amino-acid level to BiP<sup>8</sup>, suggesting that both proteins are members of a class of related molecules that promotes assembly/disassembly reactions in a range of cellular processes and responses.

In my laboratory a chloroplast protein has been implicated in the assembly of the photosynthetic CO<sub>2</sub>-fixing enzyme, ribulose biphosphate carboxylase-oxygenase (Rubisco), from its subunits<sup>10,11</sup>. The catalytic site of Rubisco is carried on the large subunit (L) which is synthesized within the chloroplast. Enzymic activity appears only after combination with equal numbers of small subunits (S) to form the L<sub>4</sub>S<sub>6</sub> oligomer; these small subunits are imported across the chloroplast envelope after synthesis in the cytoplasm. Large subunits, newly synthesized by intact isolated chloroplasts, are bound non-

covalently to an abundant soluble protein termed the Rubisco large subunit-binding protein. Bound large subunits subsequently co-migrate with the Rubisco oligomer on treatment of stromal extracts with MgATP and this transfer is inhibited by the addition of antibodies to the binding protein<sup>12</sup>. The binding protein is itself oligomeric and consists of two types of subunit in the form  $\alpha$ ,  $\beta$ . These subunits are synthesized in the cytoplasm in precursor form and imported into the chloroplast (see figure).

Rubisco is an important target for genetic engineering because of its significant influence on plant productivity, but attempts to produce mutant forms of the protein from higher plants by expressing cloned genes in *Escherichia coli* have so far been prevented by the failure of the large subunits to assemble with small subunits to produce the active enzyme. We are trying to characterize the binding protein subunits so that their DNA sequences can be expressed in the same *E. coli* cells as the Rubisco large and small subunits; in this way we hope to rescue the assembly of Rubisco and permit the mutagenic studies to proceed.

As reported at the meeting, DNA sequences encoding the  $\alpha$ -subunit of the Rubisco large subunit-binding protein have been isolated from wheat and castorbean expression libraries<sup>13</sup>. Sequence comparisons reveal amino-acid homologies of 50 per cent to two bacterial proteins of unknown function, whereas antibodies raised against the binding protein from *Pisum sativum* cross-react weakly with proteins of similar molecular mass in a range of bacterial species. The binding

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A TRIPLE sunrise over the British Antarctic Survey's site at Halley Bay. The remarkable multiple images are generated by reflections in ice crystals suspended in the atmosphere. It was from this site that significant springtime depletions of ozone in the Antarctic stratosphere were first detected in 1985. On pages 408 and 411 of this issue, P. Solomon and co-workers report microwave measurements from last year's US expedition to McMurdo Sound to investigate the 'ozone hole'. (This was discussed by J.J. Margitan in a News and Views article in *Nature* 325, 297-298; 1987.) The new results give crucial support to the idea that anthropogenic chlorine is playing a significant role in the depletion. Laboratory measurements by A.J. Hills *et al.* (on page 405 of this issue), indicate that chemical cycles involving the radicals ClO and BrO could also have an important effect. A detailed discussion of the implications of all the results from that expedition will appear in News and Views shortly (courtesy of J.C. Farman). □

protein from chloroplasts could thus have evolved from the general class of molecular chaperones specifically to mediate Rubisco assembly. Such an origin would account for the immunological and sequence similarities between the binding protein from higher plants and certain bacterial proteins.

If this general argument for molecular chaperones is correct, there may exist other proteins involved transiently in the assembly of oligomeric proteins, especially where the constituent subunits originate in different subcellular compartments, where problems of folding, transport and pool sizes are more acute. I recommend the use of non-denaturing gradient polyacrylamide gels to detect the involvement of binding proteins in the assembly of other oligomeric proteins, because this technique permits the detection at high resolution of non-covalently linked protein complexes. □

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## DNA technology

## High-altitude walking with YACs

Kim Nasmyth and John Sulston

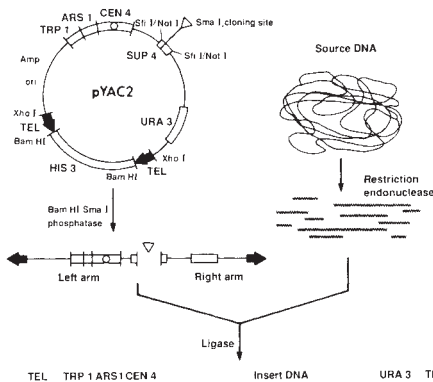
THE ability to clone and manipulate DNA from any source by ligating restriction fragments into phage or plasmid vectors which are then replicated in *Escherichia coli* underlies many significant advances in molecular biology during the past decade. One of the consequences of this recombinant DNA revolution has been that we have entered an era in which progress is now limited rarely by available technology but more often by its application to new problems. Nevertheless, two developments have pinpointed a crucial gap in the repertoire of what has now become conventional recombinant DNA technology. The first is the discovery that many eukaryotic genes are encoded by enormous tracts of DNA; and the second is a recent enthusiasm for mapping, and ultimately sequencing, entire genomes. A major impediment to studying large genes and to long-range mapping has been the small (40-kilobase) capacity of existing cloning vectors. A significant step towards resolving this limitation has just been made<sup>1</sup> by Burke, Carle and Olson, who have cloned DNA fragments an order of magnitude larger than previously possible.

That genes are encoded by long sequences of DNA is true in invertebrates and vertebrates. For instance the three genes of the bithorax complex, which specify the identity of segments 5 to 14 in the fruitfly *Drosophila*, span well over 300 kilobases<sup>2</sup>. Even more staggering is the case of the muscular dystrophy gene in man which is encoded by nearly 2 megabases (see the recent discussion in News and Views<sup>3</sup>). In other cases, for example the *engrailed* gene of *Drosophila*, regulatory rather than coding sequences are apparently spread over great distances<sup>4</sup>.

There have been several recent advertisements devoted to the subject of mapping the human genome. Rather less visible has been the appearance of a few research reports on the mapping of smaller genomes<sup>5-8</sup>. Genome mapping, which refers to the production of a completely overlapping and ordered set of clones that covers an entire genome, occupies an intermediate position between the production of unordered clone libraries and complete genomic sequences, which are still some way off for eukaryotes. The principal reason for mapping genomes is that, as the alignment between the genetic map and the genomic map of an organism proceeds, our ability to move from a genetic locus to a clone or vice versa is enhanced more and more until ultimately the two sorts of map become synonymous. Genome mapping

has therefore become crucial not only for the tracing of genes corresponding to disease loci in man but also for the cloning of developmental genes in *Drosophila* or the nematode worm, where only the locus and not the product of such genes is known.

One attempt to mitigate the small capacity of existing cloning vectors was the invention of 'leaping', in which a large DNA fragment is deleted so as to leave only its ends attached to a vector; the resulting molecule is small enough to be amplified by cloning, and can be used to identify, by hybridization, genomic sites spaced apart by the length of the original large fragment<sup>9,10</sup>. Another development was the observation by Schwartz and Cantor<sup>11</sup> that large fragments (up to 1 megabase or more) can be separated by electrophoresis in an agarose gel provided that the direction of the electric field is changed periodically (a technique called pulsed-field gel electrophoresis). The original design has been improved by Carle *et al.*<sup>12</sup> and by Chu *et al.*<sup>13</sup>. A third development was the discovery of rare cutting restriction enzymes (*NotI* and *SfiI*), whose sites are, on average, spread several hundred kilobases apart in most



Yeast artificial chromosome (YAC) cloning system. In the diagram of the vector pYAC2, pBR322-derived sequences are shown as a thin line. *SUP4*, *TRP1*, *HIS3* and *URA3* are yeast genes: *SUP4* is an ochre-suppressing allele of a tyrosine transfer RNA gene that is interrupted when exogenous DNA is cloned into the vector; *TRP1* and *URA3* are present in the artificial chromosomes and allow selection for molecules that have acquired both chromosome arms from the vector; *HIS3* is discarded during the cloning process. *ARS1* and *CEN4* are sequences that are naturally adjacent to *TRP1* on yeast's chromosome IV: *ARS1* is an autonomous-replication sequence while *CEN4* provides centromere function. The *TEL* sequences are derived from the termini of the *Tetrahymena* macronuclear ribosomal DNA (rDNA) molecules. pYAC2 can be grown as a plasmid in *E. coli*. (From ref. 1, copyright AAAS, courtesy of M.V. Olson).

genomes. Schemes based solely on these techniques, though capable of yielding valuable information, suffer from difficulties of verification because the large DNA fragments can never be cloned directly.

The recent paper<sup>1</sup> by Burke *et al.* changes this situation. It describes a breakthrough that allows the routine cloning of half-megabase-sized DNA as artificial chromosomes in yeast (abbreviated to YACs). One of the more spectacular recent fruits of work on the molecular biology of yeast is the definition of all the fundamental components that constitute a eukaryotic chromosome<sup>14</sup>. These are: *ARS* elements, required for autonomous replication of any exogenous DNA and thought to act as specific replication origins; centromeres, causing the disjunction both of sister chromatids at mitosis and of homologues at meiosis I; and telomeres, required for complete replication of linear molecules. Although each of these three elements is no more than a few hundred base pairs long, they are sufficient for almost normal chromosome behaviour when combined *in vivo* on molecules which are 50 kilobases in size or larger<sup>15</sup>. Such YACs were initially generated in yeast by homologous recombination *in vivo*, a process which by definition is unsuitable for cloning foreign DNA.

Although the potential of YACs for carrying large tracts of exogenous DNA has been apparent since they were first described, it has until now been uncertain whether it would be possible to make them completely *in vitro*, to transfer them efficiently to yeast, or whether once transferred they would be sufficiently stable for practical purposes. The results reported by Burke *et al.* dispel most of these doubts. The authors describe the construction of a vector which can be replicated as a plasmid in *E. coli*, cleavage of which yields a left chromosome arm including a telomere, a centromere and a selectable *TRP1* gene, and a right chromosome arm including a telomere and a selectable *URA3* gene. These two arms can then be ligated onto large fragments of heterologous DNA and the resulting tripartite recombinants transferred to yeast by more-or-less conventional transformation techniques (see figure). Non-recombinant chromosomes (ones that lack an insert) are avoided as the cloning site separating the two arms sits within a suppressor transfer RNA gene whose lack of function resulting from DNA insertion can be selected for. Using human DNA in the size range 50–700 kilobases, Burke *et al.* describe the generation of several hundred YACs per microgram of source DNA size ranging up to 500 kilobases. The cloned fragments can be observed directly by pulsed-field gel electrophoresis.

A key question concerns the stability of recombinants and whether clone banks are representative. The recombinants

analysed so far seem stable but it will be some time yet before we know whether all fragments are equally clonable. There are, however, grounds for optimism. Many of the types of sequences that are difficult or impossible to clone in *E. coli*, such as perfect palindromes and dispersed repetitive sequences, already exist in yeast chromosomes: eukaryote DNA segments can now be grown in their 'natural' environment as chromatin, at low copy number. Few if any mammalian genes can be spliced correctly in yeast, so YACs are unlikely to generate deleterious products.

Clearly, libraries of YACs containing DNA from humans or other eukaryotic sources will accelerate all kinds of projects, from the specific tasks of walking towards disease loci or through centromeres to more ambitious plans for the global mapping of entire genomes. By way of illustration, consider the techniques involved in mapping. Existing protocols start with a method for determining whether a given pair of clones overlaps. Initially, randomly chosen clones are analysed, but when the rate of joining becomes unacceptably low, some form of probing is used to find the desired bridging clones. A major problem is that existing cloning techniques often do not yield sufficiently random libraries for efficient mapping, so that the transition from random assembly to probing must occur at an earlier stage in the project than expected.

Cloning in yeast potentially offers, at a stroke, two huge advantages. The first is purely arithmetic: by the direct application of existing technology<sup>5-8</sup>, it should be possible to tackle projects an order of magnitude larger than the current ones. The second is that, for the reasons given above, the yeast cloning system may provide a better (or at least a different) representation of eukaryotic genomes than do bacterial cloning systems.

In addition to effecting a quantitative reduction in the enormity of mapping projects, YACs should effect a qualitative change in our ability to manipulate large stretches of DNA for functional studies. The existence of half-megabase-sized pieces of DNA should allow even longer continuous stretches to be built by homologous recombination between overlapping clones within yeast. Moreover, irrespective of the size of such chromosomes, it will be trivial to manipulate them at will using the powerful 'transplacement' techniques which are standard practice in yeast genetics<sup>16-18</sup>. These techniques enable the exact replacement of any sequence within a chromosome by copies that have been manipulated and mutated in standard *E. coli* vectors.

The main question mark over this area of application is how easy it will be to transfer YACs back into their rightful host to test their function. However, provided that they can be recovered and transferred

into the recipient, one suspects that these pieces of DNA will be as avidly incorporated into mammalian chromosomes as all other exogenous offerings. Moreover, their large size may ensure that the genes contained in them are immune from the 'position effects' that often frustrate the functional analysis of transfected DNA.

Exactly how these huge DNA fragments, an order of magnitude larger than previously available clones, can best be deployed will provide scope for much ingenuity. There is no doubt that they will be crucially important. □

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## Biogeography

# Snails and the Irish question

Peter D. Moore

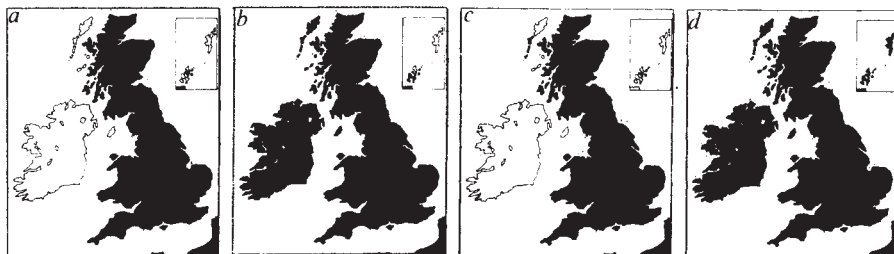
PERUSAL of distribution maps of plants and animals in western Europe has led biogeographers to regard Ireland as a particularly thorny problem. There are two basic problems conspicuous in Irish biogeography: why are there so many organisms present that one might not expect, often having their main distributions in the Mediterranean region (the Lusitanian element); and why are so many organisms absent that one might reasonably expect to be present? The answer to both questions hangs on a third question: when did Ireland become an island? Geomorphologists have strongly held and varied views, but a recent analysis<sup>1</sup> of land snail shells from a calcareous tufa deposit adds some unexpected fuel to the debate.

Preece, Coxon and Robinson have analysed<sup>1</sup> in detail slightly more than a metre of tufa exposed by road workings at Newland Cross, County Dublin, and document a sequence of plant and animal fossil assemblages. Preservation of mollusc shells is good and the authors extracted and identified more than 17,000; they also analysed ostracods and found tooth fragments of a single mammal, the wood mouse (*Apodemus sylvaticus*). It was also possible to extract and count some pollen grains from the tufa, an unusual achievement, for calcareous conditions

are not favourable for the preservation of pollen. Even more valuable was the survival of some fruits of hemp agrimony (*Eupatorium cannabinum*), which can provide samples of contemporaneous organic material for radiocarbon dating by accelerator mass spectroscopy. To add to their good fortune and to complicate the picture, Preece *et al.* also found a Mesolithic flint flake in their profile.

The results show that the tufa began forming in the early post-glacial, about 9,700 years before present (BP), and stopped growing about 7,000 BP. The pollen record shows an initial shrub tundra with juniper and grasses, which became colonized first by birch, then hazel and finally by oak and elm. During the phase of hazel expansion, somewhere between 8,000 and 7,000 BP, there was some woodland recession and an expansion of grasses. Because this event coincides with the stratified flake and was followed by the decline of the shade-demanding mollusc *Discus rotundatus*, it is reasonable to conclude that the pollen data record some effect of Mesolithic people on the local vegetation.

But the record of the tufa raises some of the old biogeographical questions for which Ireland is well known. First, the wood mouse, although now widespread in Ireland, is generally supposed to have



Distributions of a, common shrew (*Sorex araneus*); b, pygmy shrew (*S. minutus*); c, field vole (*Microtus agrestis*); and d, wood mouse (*Apodemus sylvaticus*) in the British Isles. (From ref. 1.)



been introduced by man<sup>2,3</sup>. Berry<sup>4</sup>, in his study of genetic relationships among wood mice of the Hebrides, suggests that Vikings transported this mammal, perhaps as far as Ireland. But the new data imply that the wood mouse is native to Ireland, and raise the problem of how it arrived there. Jared Diamond, in his recent News and Views article<sup>5</sup>, provides several possible answers to this question.

The analyses of molluscs are equally interesting. In general such analyses reflect the changing climate and vegetation documented by the pollen stratigraphy, but some of the woodland species, such as *Discus rotundatus*, were late arriving, perhaps even after 8,000 BP. Could such species have been brought across the Irish Sea by Mesolithic populations? Did these molluscs arrive under their own propulsion, or were they pushed? The answer depends on when Ireland became severed from mainland Britain as the rising post-glacial sea level submerged any residual land bridge or bridges. If a land bridge had persisted until about 6,000 BP, it could account for the migration of some temperate mammals, such as the pigmy shrew<sup>6</sup>, and even those invertebrates not noted for their migrational alacrity, such as land snails, could have made it. But if this was the case, we are left with the question of why the common shrew, mole, weasel, polecat, roe deer, beaver and the voles all failed to reach Ireland.

Sadly, geomorphological studies fail to produce a definitive answer as to when the final breach was made. The latest review of the evidence<sup>7</sup> concludes that a southerly connection (between perhaps Cornwall or Wales and south-east Ireland) is out of the

question. Syngé<sup>8</sup>, however, believed that a southerly bridge existed in late-glacial times (linking to the Lleyn Peninsula in North Wales), but would have been breached soon after 12,000 BP, allowing only a short period for a temperate fauna to immigrate at the beginning of the late-glacial interstadial. Devoy<sup>7</sup> believes a northerly bridge to be the only possibility, linking northern Ireland with south-west Scotland, but suggests that it was in operation only between about 11,400 and 10,200 BP. Because the polar front is thought to have been in a southerly position in the North Atlantic at this time<sup>9</sup>, perhaps as far south as Iberia, there is little opportunity in this scheme for the immigration of a temperate biota.

Just two biogeographical explanations remain available to account for the information emerging from Newlands Cross. Either several species must have survived the last glacial maximum in some sheltered corner of southern Ireland; or the Irish Sea was not an insurmountable barrier to migration and snails are more mobile than is usually supposed. □

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## Astronomy

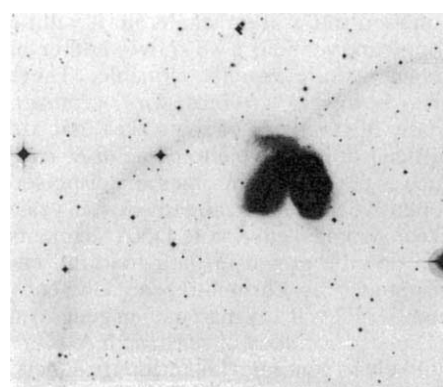
# Warps, galaxies and haloes

R.D. Davies

SOME galaxies are warped, showing large-scale distortions in their outer regions. The small fraction of galaxies (about 1 per cent) that show this intriguing peculiarity belong to the class of spiral galaxies that generally have most of their stars in a planar disk. A warped galaxy seen edge-on is shaped like an integral sign in which the two extremities curl away from the opposite sides of the disk. Although warped galaxies have been recognized in deep-sky photographs for the greater part of this century, radio studies using the 21-cm line of neutral hydrogen (H I) during the late 1950s showed that our own Galaxy, the Milky Way, is also warped. Gas in the northern constellation Cygnus lies 1 kpc (one tenth of the distance from the Sun to the centre of the Milky Way) above the galactic plane, and gas in the southern constellation Carina lies a similar distance

below the plane. The largest warp so far detected in a galaxy is described by Bottema, Shostak and van der Kruit on page 401 of this issue.

What causes such warps? One answer is evident from photographs that often show a close companion interacting with the warped galaxy; the companion may also show a disturbed morphology. It is not necessary for the two galaxies to have interpenetrated for them to be warped. Indeed a much more symmetrical distortion of a galaxy is produced by the tidal interaction of a close, but non-penetrating, companion orbiting at an appreciable angle to the rotation plane of the warped galaxy. Detailed model simulations by A. Toomre and J. Toomre (*Astrophys. J.* **178**, 623-666; 1972) of tidal (gravitational) interactions between galaxies could reproduce the structures seen in the



The two neighbouring galaxies of the Antennae, NGC 4038/39 (negative optical photograph). The long limbs to the left and right of the two galaxies are caused by their mutual gravitational interactions. (Courtesy of the Royal Observatory, Edinburgh.)

photographs. Such fascinating double systems as the Mice (NGC 4676) and the Antennae (NGC 4038/39) with long tidal plumes could be readily explained in terms of close tidal interactions with carefully chosen orbital parameters. More distant encounters could produce the less disturbed but more symmetrical tidal warps.

The subject of warps was given a new impetus when 21-cm H I maps were made of a number of edge-on spiral galaxies (for example, Bosma, A. thesis, Univ. Groningen; 1978). These showed warps in the H I regions lying well beyond the optical edges of the galaxies, even when the optical image showed no, or a very small, warp. Again this could be understood in terms of a tidal effect upon the H I gas which can be traced further from the centre of a galaxy than the light; the further the material is from the centre, the greater the tidal displacement. There is an apparent snag in this picture: not all the warped galaxies have a large, near companion to produce the observed tidal effect. Other explanations have been sought for these field-galaxy warps. They could conceivably be produced by the internal mass distribution of the galaxy if there were significant mass asymmetries about the plane of rotation; such asymmetries are known to occur in the well-known central bars of galaxies and may occur in the triaxial (ellipsoidal) haloes of dark, hidden matter surrounding the visible disks of spiral galaxies (Sparke, L. *Mon. Not. R. astr. Soc.* **211**, 911-926; 1984). Alternatively, a warp could be produced by a dark companion or by the capture of gas from the intergalactic medium — both rather extreme hypotheses. Small companions between 0.01 and 0.1 of the mass of the galaxy, if placed in a suitable orbit, could produce these outer warps.

The detection of warps is not restricted to edge-on galaxies. They can be detected through their kinematic effects even in almost face-on spiral galaxies. The 21-cm H I observations have shown antisymmetrical velocity distortions in several

nearby galaxies (Cohen, R.J. *Mon. Not. R. astr. Soc.* **187**, 839–845; 1979; Appleton, P.N. *et al. Mon. Not. R. astr. Soc.* **221**, 393–407, 1986). These observations are consistent with large-scale warps involving motion out of the plane and oppositely directed at two extremities of the galaxy.

The galaxy (NGC4013) reported by Bottema *et al.* in this issue shows a particularly spectacular warp, extending about 50 per cent beyond the optical image and very symmetrical. The warp shows up so clearly in part because it does appear to be more pronounced than any previously observed, and also because the geometry of NGC4013 and its companion is especially favourable for observation from Earth. There are two candidate galaxies that could be causing the perturbation, both at the same distance from, and on opposite sides of, NGC4013. One is

NGC3938 and the other NGC4051, a well-known active Seyfert galaxy. It is interesting to note the growing view that active galaxies are the result of such interactions. The other important deduction from the new H I observations of NGC4013 relates to its invisible but massive halo. The warp actually begins where the visible stellar disk tapers off; moreover the kinematics of the H I rotation curve shows mass throughout the H I region. The warp therefore appears to lie in an extended halo of hidden matter. Undoubtedly, this exemplary galaxy will be thoroughly scrutinized as astronomers try to unravel the detailed kinematics of warping and the role of massive haloes. □

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## Vision

# How do birds accommodate?

Graham R. Martin

TO SEE this print clearly and then to look away and see a distant object equally well requires a change in the focal length of the eye's optical system. This accommodative change in the refractive power of our eyes is brought about by muscles that alter the curvatures of the lens surfaces of the eye. With age, the lens becomes more resistant to deformation and the ability to accommodate diminishes. But how do other vertebrates, such as birds, accommodate? After many years of doubt, David Troilo and Josh Wallman of the City University of New York<sup>1</sup>, and Frank Schaeffel and Howard Howland of Cornell University<sup>2</sup> now present convincing evidence that corneal accommodation occurs in pigeons and chickens.

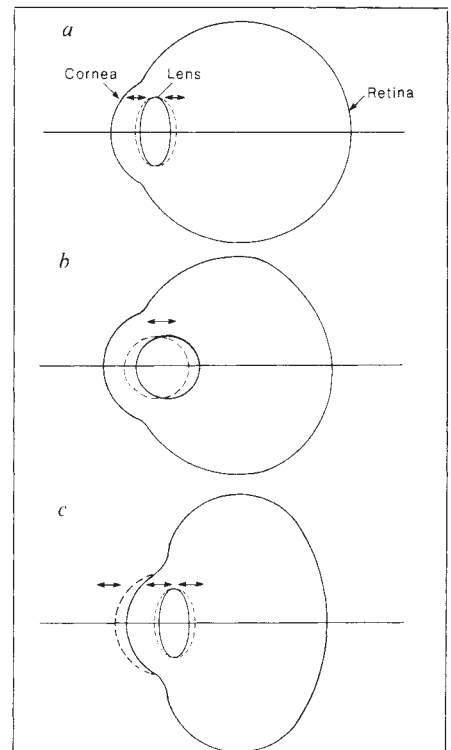
The mammalian accommodation system of changing the refractive power of the lens (*a* in the figure) is only one option for altering the power of a vertebrate eye. The optical system consists of two principal components, the cornea and the lens, and the refractive power of the whole system can be altered by varying the power of either component or by altering their separation. The latter option is the accommodative mechanism found in some fishes and also recently demonstrated in anuran amphibians<sup>3</sup>. In these eyes, a lens of fixed focal length is moved towards or away from the retina in much the same way that the lens within a microscope or camera can be brought to focus objects at various distances (*b* in the figure). But what about altering the refractive power of the cornea? The cornea is in essence a simple convex lens whose refractive power is produced by the curvature of its surface which separates two media of different refractive

index. Increasing its curvature would increase the cornea's refractive power.

In the mammalian eye there is no evidence that this occurs; indeed there are no intraocular muscles that could bring about such a change. The bird eye, however, does contain a set of muscles that could alter corneal curvature (*c* in the figure). Crampton's muscles are anchored around the corneal margin and also to a ring of bones within the eyes, the scleral ossicles, which could provide a firm base for the muscles to pull against.

Evidence that birds do indeed accommodate by changing the curvature of their cornea was first presented as long ago as 1892 when Beer<sup>4</sup> observed the movements of pins placed in the corneas of a range of species including pigeon, chicken, ducks, hawks and owls. Corneal accommodation in the pigeon was described again by Gundlach<sup>5</sup>, but by modern standards the evidence from both studies was not convincing. The problem was held in abeyance until recently, when more systematic studies found no corneal accommodation in owls, ducks and pigeons<sup>6,8</sup>.

Unlike previous studies that attempted to induce accommodation *in vitro*, Troilo and Wallman<sup>1</sup> used *in vivo* electrical stimulation of the Edinger–Westphal nucleus of the chick brain which induces accommodation through the normal neural pathway and does not interfere directly with the eye. Schaeffel and Howland<sup>2</sup> used intact animals and monitored accommodation with a modified retinoscope and measured the curvature of the cornea with a keratometer. They induced accommodative changes simply by presenting the birds with an interesting



Different types of accommodation mechanisms found in vertebrate eyes. *a*, In mammals, including man, the curvature of the lens surfaces is changed to increase the power of the lens only. *b*, In fish and anuran amphibians a lens of fixed focal length is moved towards the cornea, whereas in birds (*c*) the curvature of both the lens surfaces and of the cornea is altered, thus changing the refractive power of both lens and cornea simultaneously.

target, such as food, at close range. Both techniques provide clear evidence of corneal accommodation and also demonstrate that in the bird eye corneal and lenticular accommodation occur in tandem, with the corneal component having a proportionately greater role in the lower range of accommodation.

Thus, accommodation in birds is now thought not only to involve a different mechanism to that of our own eye but also seems to be considerably more complicated, as it involves simultaneous alteration of the power of the eye's two principal refractive components. How coordinated neural control of these two accommodative components is achieved is clearly of interest, as is the extent to which these results apply to other species. □

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## Mathematics

# The battle of the biquadrates

Ian Stewart

ADVANCES in mathematics take many forms. Entire new subjects can arise from a single brilliant idea. At the other extreme, long-standing problems can crumble under a sufficiently powerful attack. The metaphor is apt: research and war have much in common. Some problems are stormed by force of arms and superior generalship, some are devastated by new weaponry, some exist in a permanent state of siege. And some surrender only after a lengthy war of attrition.

Waring's problem (see my News and Views article in *Nature* **323**, 674; 1986) falls into this last category. In his *Meditationes Algebraicae* of 1770, Edward Waring stated without proof that every positive integer is a sum of at most 9 cubes, 19 biquadrates, "and so on". One case of Waring's problem, that for biquadrates — fourth powers — has finally been resolved by the collaborative work of Ramachandran Balasubramanian at the Institute of Mathematical Sciences, Madras and Jean-Marc Deshouillers and François Dress at the University of Bordeaux. In two short notes (*C.r. Acad. heb. Séanc. Sci., Paris* **303**, 85–88; 1986 and **303**, 161–163; 1986) they show that, for fourth powers, Waring was right.

Waring was presumably led to his conjectures by numerical experiments. For example the number 79 can be written as  $4 \times 2^4 + 15 \times 1^4$ , involving 19 fourth powers in all. Because the only fourth powers less than 79 are 1 and 16 it is very easy to see that 79 cannot be written using 18 fourth powers or fewer. Thus the maximum must be at least 19 fourth powers, and it remains to show that no number requires more than 19. In 1974, H.E. Thomas (*Trans. Am. math. Soc.* **193**, 427–430; 1974) showed that at most 22 fourth powers are needed. Thus the correct number lies somewhere between 19 and 22. The new work reduces the upper bound from 22 to 19, closing the gap completely.

## Proof

The proof falls into two distinct parts. In the first, it is shown that every sufficiently large integer is a sum of 19 fourth powers. In the second, the remaining cases are disposed of. This is often a sound strategy in number theory, for reasons that would be not unfamiliar to a sociologist: small numbers often behave in exceptional ways, whereas large numbers are usually more predictable. (Against this remark we must set the celebrated proof that all numbers are exceptional; if not, there would be a smallest unexceptional number, which, by

its very definition, would be exceptional.)

Such is the strategy, but it is not always easy to carry it out successfully. In the case of Waring's problem for biquadrates, sufficiently large means "greater than  $10^{367}$ ". That is, Balasubramanian and his colleagues prove by one method that every number with 367 digits or more is a sum of 19 fourth powers, and then deploy new forces to mop up the stragglers with a mere 366 digits or less.

The first step may be considered classical: it is the circle method of G.H. Hardy, J.E. Littlewood and I.M. Vinogradov. The original number-theoretical question is turned into one in complex analysis, and solved by powerful analytical techniques. To understand how the analysis enters, it is convenient to start with an easier problem: Lagrange's theorem that every positive integer is a sum of four squares. This is just 'Waring's problem' for squares, but Lagrange got there first. Consider the generating function  $(1+z+z^4+z^9+z^{16}+z^{25}+\dots)$ . Expand this as a power series in  $z$ . Then a little thought shows that the coefficient of  $z^N$  is precisely the number of different ways in which  $N$  can be written as a sum of four squares. If it were possible to prove, analytically, that every such coefficient is greater than zero, then we would have an analytical proof of Lagrange's four-squares theorem.

Waring's problem for fourth powers can be attacked similarly, using the generating function  $(1+z+z^{16}+z^{81}+z^{625}+\dots)^{19}$ . The coefficient of  $z^N$  now gives the number of ways in which  $N$  can be written as a sum of 19 fourth powers, and again we have to show it is greater than zero. To pick out a single coefficient we effectively apply Fourier analysis. Multiply the generating function by  $z^{-N}$ ; replace the variable  $z$  by  $e^{ia}$ , a general point on the unit circle in the complex plane; and integrate round the circle with respect to  $a$ . All terms vanish except one — this is what makes Fourier analysis tick — and that term is the required coefficient. In brief, there is a rather complicated integral whose value is the number of ways in which  $N$  can be represented as a sum of 19 fourth powers, and we have to prove that it is greater than zero.

The Hardy–Littlewood–Vinogradov circle method tackles this by dividing the circle into two subsets, the major and minor arcs. On the major arcs,  $a$  can be well approximated by a rational number with small denominator; on the minor arcs it cannot. It is then shown that the integral over the major arcs is so large that the integral over the minor arcs cannot cancel

it out. Thus the total integral is non-zero.

The method is not easy to use: the choice of major and minor arcs is crucial, and less than obvious, and it is not straightforward to estimate the sizes of the two integrals. In the earlier work of Thomas, the estimation of the integral over the major arcs took 40 pages and several computer calculations. Balasubramanian *et al.* use an idea from probability theory to obtain, in only half a page, an estimate that is almost as accurate — and good enough for the proof to be carried through. Their main effort is expended on the minor arcs, and while it follows the traditional strategy it contains several innovations. In particular they use an idea of R.C. Vaughan (*Camb. Tracts Math.* **80**, 1981), in which the traditional choice of major arcs is modified by making them slightly larger: this makes the difficult minor arcs smaller and simplifies some of the arguments.

## Victory

This completes step one: every integer with more than 367 digits is a sum of 19 fourth powers. The large number of digits may seem surprising, but it is necessary to make the estimates valid. Large numbers like this are common in analytical number theory because it is often  $\log(N)$  or even  $\log(\log(N))$  that must be large rather than  $N$  itself.

Entirely different weaponry is now brought to bear to mop up the stragglers. In fact the authors give themselves some room to manoeuvre by proving that every integer with less than 378 digits is a sum of 19 fourth powers. Of course, in principle this could be achieved by trial and error on a computer, but the number of digits is so great that the battle of the biquadrates would be interrupted by Armageddon.

So something more subtle is needed. The main idea is to find a sufficiently big set of numbers which, save for rare exceptions, are sums of a mere five fourth powers. These provide a bridgehead from which an invasion can be mounted on the remaining numbers, by deploying only a further 14 fourth powers. Finding this set of numbers took 150 hours on a main-frame computer and needed special tricks to reduce the demands on memory capacity. The invasion on the remaining numbers uses a variation of the 'greedy algorithm': start by subtracting the largest fourth power available and work on what is left.

Thus the 216-year war of attrition against Waring's problem for fourth powers has ended in victory. The battle of the biquadrates is won, VB Day is declared, mathematics is triumphant. But what of cubes, fifth powers, sixth powers . . . , googolplexiquates? The greater conflict continues. Cry 'havoc!' and let slip the dogs of Waring. □

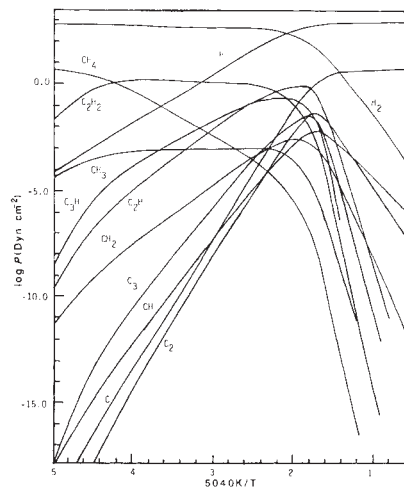
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## Diamond formation in carbon-star atmospheres

SIR—Lewis *et al.*<sup>1</sup>, from electron-microscopic examination of HF/HCl-insoluble residues of the Murchison (type C2), Murray (C2), Allende (C3V) and Indarch (EH4) meteorites, have discovered diamond grains typically 50 Å in size and in concentrations up to 400 p.p.m. Mass spectrometry revealed Kr and Xe to be enriched twofold in the lightest and heaviest isotopes, <sup>15</sup>N depleted relative to terrestrial standards, and to have <sup>13</sup>C within the terrestrial range. They argued that the diamonds are of extra-solar origin, with hydrogen, nitrogen and the noble gases having been ion-implanted during a supernova event after ejection of the grains from a red giant or planetary nebula, and that laboratory experiments that produce diamond films from the gas phase<sup>2</sup> support a stellar-atmosphere origin.

I have examined this hypothesis to determine whether it is consistent with stellar atmosphere models. Calculations of the equilibrium abundance of molecular species in carbon-star atmospheric conditions<sup>3</sup> (see figure) show a decrease in atomic hydrogen abundance at temperatures below 3,150 K. Also in this temperature range, many carbon molecules (CH, CH<sub>2</sub>, CH<sub>3</sub>, C<sub>2</sub>, C<sub>2</sub>H, C<sub>2</sub>H<sub>2</sub>, C<sub>3</sub>, C<sub>3</sub>H) are formed. This is interesting because the stellar atmospheric pressure,  $P_g = 10^3$  dyn cm<sup>-2</sup> = 0.75 torr, and composition ratio, H/C = 200, are comparable with those of diamond-film-forming experiments by vapour deposition, in which atomic hydrogen appears to be necessary<sup>2</sup>. A gradient in hydrogen-atom concentration is to be expected in both the laboratory experiments and stellar atmospheres. In the laboratory this gradient exists because of the association of hydrogen atoms into molecules at the substrate surface, resulting in hydrogen-atom depletion in a layer near the surface and diamond film formation on the substrate. In the stellar environment the gradient in hydrogen-atom concentration would be a consequence of a thermal gradient and the relation given by the equilibrium calculation<sup>3</sup> shown in the figure.

The envelope of an evolved star is convective, causing thermal (and atomic hydrogen) gradients across the surface as well as radially. As pressure scale-heights are comparable to the stellar radius, the number of convection cells is ~10–100. Large temperature differences of the order of 1,000 K can exist over the stellar-atmosphere surface area, resulting in patches of grain formation<sup>4</sup>. The distribution, across the stellar surface, of the convective cells would be expected to change on timescales of 200 days, resulting in temperature variation other than that due to radial motion. The constantly



Atomic and selected molecular abundances in the atmosphere of a giant carbon star (ref. 3). The H/C abundance ratio is 200.

changing temperature of a parcel of gas will result in variable hydrogen-atom partial pressures, with the formation of diamond grains when conditions are optimum.

What remains to be established is the mechanism by which diamond grains are nucleated by a decrease in hydrogen-atom partial pressure. Experiments directed at the formation of grains (as opposed to films) are desirable. The task might be accomplished by passing a gas mixture with H<sub>2</sub>/CH<sub>4</sub> = 200 through a tungsten tube at 2,000 °C, and taking the stream out

through a temperature gradient established along an attached sapphire tube that is radiatively cooled along its length. Evidence for particle formation as diamond smoke would be sought by observing through the sapphire.

Wright and Grady<sup>5</sup> have suggested that, because the <sup>13</sup>C/<sup>12</sup>C ratio of the meteoritic diamonds is not very different from terrestrial values, one should examine a Solar System origin. This is reasonable, because diamond formation on mineral grains in the Solar System is more like what has been demonstrated in the laboratory. For a pre-solar origin, conditions in the solar nebula, or at least the local conditions in which the diamond material of the four meteorites consolidated, would have had to be similar to a carbon-star atmosphere in pressure, temperature and composition. Perhaps comets falling into the solar nebula could have provided these conditions on a local scale. This work was supported by NASA.

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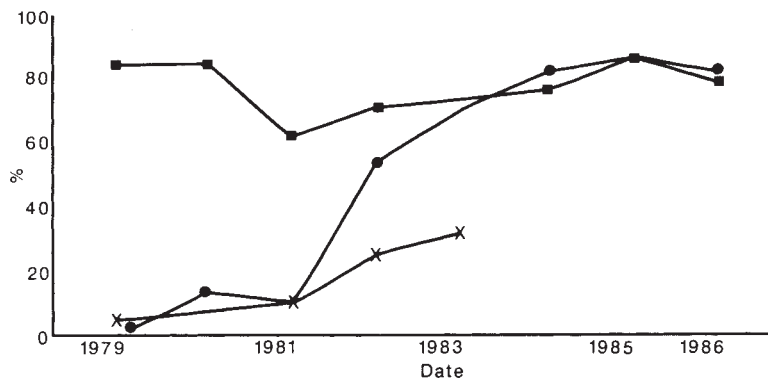
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## Italian HIV infection updated

SIR—In order to understand the epidemiology of AIDS (acquired immune deficiency syndrome), simple mathematical schemes are needed to clarify the meaning of many parameters which may influence the dynamics for transmitting HIV (human immunodeficiency virus). In their recent article, however, May and Anderson report some unclear findings<sup>1</sup>. The data indicate the rise in HIV seropositivity in two Italian cities as representative of Italy and compared them to those in

London, San Francisco and New York. In addition, data on Italian drug addicts are compared with those from homosexual cohorts, even though the article seems to stress the necessity of collecting clear information in selected groups sharing a certain homogeneity in their behaviour. It is incorrect to compare a homosexual HIV transmission plot with an HIV intravenous route, which probably occurs in the majority of drug addicts.

This, however, is not the main uncertainty emerging from the article. In Italy and Spain drug addicts account for 51% and 46% respectively of the total number



Evaluation of 451 sera collected in Rome during 1979–1986, showing % seropositive. ■, Drug addicts still continuing abuse in 1987; dates indicate when abuse was started. ●, Drug addicts stopping abuse; dates indicate when abuse stopped. ×, Frozen sera stored at different times from drug addicts.



of declared AIDS cases. Only limited information is available and the rapid diffusion of HIV infection among drug addicts in large Italian cities (such as Milan, Florence and Rome)<sup>2</sup>, although showing an increasing prevalence (1981–1985), does not illustrate national differences, in particular for southern Italy.

An epidemiological study involving 160 centres in large and small cities from all over Italy and including more than 18,000 drug addicts has recently been carried out. Seropositivity was only scored when two enzyme-linked immunosorbent assay (ELISA) tests were positive. Seropositivity among drug addicts was 54% in the north of Italy, 39% in the centre and 26% in the south. Preliminary data suggest that seropositivity in drug addicts varies not only from big to small cities, but also that an unexplained geographical distribution exists<sup>3</sup>.

In order to update information on the rise of seropositivity, we evaluated 451 sera collected in Rome between 1979 and 1986, selecting people giving up drug abuse at different times (see figure). In addition, a study of 513 sera of addicts who started abusing drugs from 1981 onwards, shows that from 1983 to 1984 no evidence of behaviour modification can be observed. Data on antibodies to HIV in sera of drug addicts in Rome stored in different years before 1983 show that the evolution of seropositivity is not homogeneous in different areas of the same city. All these data demonstrate that, despite a prevention campaign, no decrease of seropositivity in new drug addicts has been observed. In addition, our data show that 15% of 460 partners of seropositive persons are also seropositive by both two ELISA tests and a Western blot test. This category represents a very high risk group for the possible extension of the infection in Italy among the general population.

Steps to prevent HIV infection in drug addicts should include recommendations not only for avoiding the sharing of syringes but also for avoiding at-risk sexual intercourse. In our opinion care and prudence are needed when partial data are used for general comparison although a common trend shared in Western countries is now evident.

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## Elemental metallic irrelevance

SIR—Regardless of the eventual outcome of the polemic concerning the role of aluminium in the aetiology of Alzheimer's disease, evidence gleaned from elemental metallic contamination of brain tissue<sup>1</sup> is likely to be as irrelevant as would be searching for improvements in rheumatoid arthritis in patients with gold threads sewn into their bodies. There are other similar examples: the infrequency of poisoning from years-long exposure to embedded ballistic lead<sup>2</sup>; and the lack of toxicity from accidentally-ingested elemental mercury<sup>3</sup>.

The chemistry of ionized metals is as different from that of their zero-valence states as should be economic considerations from the legitimate rationale for publication of scientific findings. Regardless of the market value of the stocks of manufacturers of various metal wares, the search for the culprit in Alzheimer's disease will go on.

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## Inositol phosphates and calcium entry

SIR—Neher's summary<sup>1</sup> of current thinking on "receptor-operated  $\text{Ca}^{2+}$  channels" which was inspired by Kuno and Gardner's<sup>2</sup> experiments, nicely encapsulates the apparent contradictions in the three presently proposed mechanisms as to how these channels operate. It may be, however, that the experimental results are not as contradictory as they seem.

$\text{Ca}^{2+}$ -mediated  $\text{Ca}^{2+}$  entry as shown by Von Tschärner *et al.*<sup>3</sup> is, at least in some tissues, demonstrably not the principal mechanism of receptor-mediated  $\text{Ca}^{2+}$  entry. In these tissues a complete temporal dissociation can be shown between the  $\text{Ca}^{2+}$  pulse caused by intracellular mobilization, and the sustained  $\text{Ca}^{2+}$  rise caused by  $\text{Ca}^{2+}$  entry (for example, refs 4–6). Perhaps, therefore,  $\text{Ca}^{2+}$ -stimulated  $\text{Ca}^{2+}$  entry is a supplementary mechanism, or a further modulation of inositol phosphate-mediated mechanisms. Evidence that a diffusible second messenger is controlling  $\text{Ca}^{2+}$  entry (but that the messenger is not  $\text{Ca}^{2+}$ ) is found also in the experiments of Susuki *et al.*<sup>7</sup>.

Kuno and Gardner's results<sup>2</sup>, showing an effect of  $\text{Ins}(1,4,5)\text{P}_3$  on inside-out membrane patches, are easily reconciled with our proposal<sup>8</sup> that  $\text{Ins}(1,3,4,5)\text{P}_4$  plays a key role in  $\text{Ca}^{2+}$  entry. The union of the two hypothesis lies in our experimen-

tal observations<sup>8</sup> that there is an absolute requirement for a  $\text{Ca}^{2+}$ -mobilizing  $\text{InsP}_3$  to be present in order that  $\text{InsP}_4$  can exert its biological effect. Further experiments<sup>9</sup> have shown us that we cannot substitute for  $\text{InsP}_3$  with  $\text{Ca}^{2+}$  (taking eggs of *Lytechinus variegatus* to the brink of activation with the  $\text{Ca}^{2+}$  ionophore ionomycin, does not sensitize them to  $\text{InsP}_4$ ). We interpret these data as  $\text{InsP}_4$  needing the obligatory help of an  $\text{InsP}_3$  molecule to control  $\text{Ca}^{2+}$  entry<sup>7</sup>. This strengthens our suggestion<sup>8</sup> that the two molecules are acting together, most likely by a mechanism related to that proposed by Putney<sup>10</sup> in which  $\text{InsP}_3$  indirectly controls  $\text{Ca}^{2+}$  entry because there is rapid re-filling of the  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  pool from outside the cell. But this refilling requires a  $\text{Ca}^{2+}$  pump in the endoplasmic reticulum, and such an indirect mechanism is not consistent with Kuno and Gardner's<sup>2</sup> data.

If, however,  $\text{InsP}_4$ 's role is to 'short-circuit'  $\text{Ca}^{2+}$  transport between the endoplasmic reticulum and plasma membrane<sup>9</sup> (perhaps by forming a link analogous to a gap junction), then  $\text{Ca}^{2+}$  passage through the  $\text{InsP}_3$ -controlled pore in the endoplasmic reticulum would be the rate-limiting step for  $\text{Ca}^{2+}$  entry. Such a mechanism could unite Kuno and Gardner's data with ours, assuming that some aspect of the preparation of their inside-out patches had constitutively activated the  $\text{InsP}_4$ -mediated process (for example, washing the cells, exposure to high KCl medium, or in Fig. 3 of Kuno and Gardner<sup>2</sup>, pre-treatment of the cells with PHA). This latter suggestion does not necessarily require any particular mechanism; it simply accommodates our experimental observation<sup>8,9</sup> that both  $\text{InsP}_3$ , and  $\text{InsP}_4$  are required to see a biological effect.

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## Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □

# Minsky's mentality

P.N. Johnson-Laird

**The Society of Mind.** By Marvin Minsky. Simon & Schuster: 1987. Pp.339. \$19.95. To be published in Britain on 1 September by Heinemann, £15.95.

MARVIN Minsky is the *éminence grise* of artificial intelligence. As the founder of MIT's AI laboratory and author of one of the (non-)standard texts on the theory of computation, he has for years exerted a marked influence on the field. Another book on 'perceptrons' — elementary associative networks — which he co-authored with Seymour Papert, deterred almost an entire intellectual generation from pursuing them, because it proved that there are certain simple patterns, such as exclusive disjunctions (A or else B, but not both), which perceptrons could not identify.

His positive contributions to the field are a little harder to identify: until now, he has not published any major monograph on his own research, and one tends to think of him as an advocate of certain general ideas rather than as someone who devises computer programs capable of advanced cognitive skills. Workers in artificial intelligence have a tendency to develop by metamorphosis: an early stage of intense activity yielding an impressive large-scale program gives way to a more meditative stage in which general ideas — often philosophically hostile to the earlier work — are published without benefit of computer implementation. The practical caterpillar turns into a butterfly mind: the 'hacker' gives way to the Heideggerian. Minsky, however, has always been a speculative theorist and a thread of consistency runs through his career. His remarkable new book is the outcome of his research over the past decade, and it presents his considered theory of the mind.

The poet Paul Valéry wrote, "the mind is the collective pseudonym of a crowd of very different entities", and the same metaphor underlies Minsky's theorizing. He conceives of the mind as made up from a vast set of entities, or 'agents', each of which is capable of only simple, mindless activities that require no intelligence. When they are joined up in special ways, however, the result is intelligence. Thus, when you pick up a cup of tea, your action depends on agents for grasping the cup, keeping it level, moving it to your lips and so on. There are some hundred or so agents for shaping your hand into the appropriate position. Overall, there are many millions of agents enabling you to do everything you can do. They are wired up to communicate with each other, and there is a hierarchy of control with higher-level agents calling on the services of

lower-order ones. There is also some heterarchical organization, where one agent may call on another, which in turn calls on it, in mutually recursive ways familiar to programmers.

Minsky explores a wide range of mental phenomena and offers explanations of them in terms of the activities of agents. An important role in the theory is played by a special class of agents (the so-called



Marvin Minsky — provocative ideas.

'K-lines') that make records of what other agents are doing. If you activate a particular K-line, it restores these other agents to their previous states, and so you *remember* part of your earlier mental state. As Allen Newell and Herb Simon showed 30 years ago, a computer can be programmed so that its performance is driven by the difference between the current situation and some specified goal. Minsky translates this idea into his society of agents and argues that the resulting 'difference engine' is goal-driven. He shows how such a device could underlie a variety of mental skills.

Like Minsky, most cognitive scientists believe that the mind rests on a large number of parallel computational processes, and that these processes are organized hierarchically. What is controversial is the nature of the individual processors. One school of thought, sometimes known as 'connectionism', argues that each processor carries out only a simple computation: like a neuron, it passes on a level of activation as a function of the sum of the activation that it receives. These theorists have, in effect, revived the perceptron and shown how to

represent complex patterns, such as exclusive disjunctions, by adding processors between its input and output layers. More innovatively, they have devised programs that can *learn* to adjust the strengths of connection between processors so as to represent such complex patterns. Another school of thought, particularly associated with the work of some of Minsky's colleagues at MIT — Noam Chomsky, Jerry Fodor and, in a slightly different vein, the late David Marr — argues for a set of separate large-scale computational 'modules', each specialized to a particular task, such as stereopsis or the grammatical analysis of sentences.

Minsky alludes briefly to the distributed representations used in connectionist networks, but not at all to modules, though they seem to be similar to his notion of an agency (a collection of agents). It is only in the appendix of his book that he offers what, in fact, is a sensible reconciliation of the two schools of thought. Each agency may rely on a connectionist network of its own, but the mind cannot be just one vast connectionist network. This picture, by no means unique to Minsky, looks to be the best current bet about the design of mental architecture.

If Minsky (and cognitive science) is right, the mind is a machine. Most people, as he recognizes, are offended by this idea. "Ridiculous", they say, "I don't feel like a machine, and besides I have free will". Minsky has a nice quip about the first of these claims: "if you're not a machine", he says, "what makes you an authority of what it feels like to be a machine?". As to free will, he regards it as a myth, though one that we are forced to believe in. Human beings are difference-engines with computational goals, and these goals correspond to their intentions. On this issue (and others) his claim is that it is futile to ask whether there is any difference between person and program: "We need not force ourselves to decide questions like whether machines can have goals or not. Words should be our servants, not our masters". The danger of not asking such questions, however, is that the really difficult problems may be defined out of existence. The manoeuvre cuts human beings down to the size of computers.

In fact, there is a critical difference between the two. Human beings *know* that they can behave intentionally, and they can use this knowledge in explaining behaviour and even, sometimes, in determining what they should do. Computers as they currently operate have no such knowledge; indeed, such is the poverty of their connection to the world outside them that it is debatable whether they have any knowledge at all. As to the interpretation of their computations, they leave that to



their users. Similarly, human beings — unlike existing computers (and some other living creatures) — can reflect on *how* to make a decision, and on how to make a decision amongst different methods of making a decision ... and so on. They can even elect to make an arbitrary choice that in some circumstances frees them from the constraints of rationality. Human intentionality may one day be modelled computationally, but only if theorists act like proper 'difference engines' and keep in mind the discrepancies between their theories and human mentality.

The scope of Minsky's interests is exhilarating. He has things to say about nearly all psychological topics — learning, motivation, the sense of self, vision, pleasure and pain, the meanings of words, how children develop intellectually, reasoning and problem solving, creativity and genius, consciousness, emotions, jokes, and so on and on. It is all but impossible to summarize his book because its structure, quite deliberately, mimics the structure of the theory. Each page is a self-contained 'agent' with its own title and part number. It may begin with an apposite quotation, often from an unlikely source such as Dr Johnson or Kurt Vonnegut; it elucidates some aspect of the mind making its point economically and perhaps aided by a diagram, and its moral

may be encapsulated in an italicized principle. The pages make up 30 chapters, but the organization of a chapter is manifest solely in its title and the part numbers of its component pages. There is only a brief bibliography (attached to the items in the glossary) and there are few references — often not even to work reaching similar conclusions. The format and the imprimaturs from luminaries in the world of science fiction suggest a book for the coffee-table. The contents suggest a book for the AI laboratory. The result is a unique Minskian amalgam — a coffee-table monograph, that is both readable and provocative. But is it good science?

A sceptic is likely to complain that Minsky appears neither to have checked

whether his ideas add up to a coherent theory by building computational models of them nor to have submitted them to any sort of empirical test. By his own reckoning, he has made hundreds of assumptions, and many of them may be wrong. An enthusiast will retort that Minsky is attempting to provide a coherent framework for psychology, and that until he succeeds it would be premature to try to show that his theory is better than others. Minsky's book is speculative, but, as he says, it is intended to be an adventure story for the imagination, not a scientific textbook. □

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## Social invention

*Edmund N. Todd*

**Diesel: Technology and Society in Industrial Germany.** By Donald E. Thomas, Jr. *University of Alabama Press:1987. Pp.279. \$26.95, £22.50.*

DURING the late nineteenth century, Germany underwent impressive economic and urban development, which formed an industrial state from one based on agriculture. Conservative elements were concerned with promoting the agricultural sector and damping down what they considered to be unhealthy industrial concentration. The growth of a large Marxist party representing the industrial working class also threatened traditional aristocratic and new industrial élites. As social and economic structures changed, German engineers developed their profession, modelling it on the older legal profession, which was closely tied to the conservative bureaucracy.

Although technological change threatened the old order, engineers hoped to increase their professional status by claiming a cultural mission of mediating between labour and capital and by promoting further technological change to preserve the old order. For instance, many engineers sought to develop new sources of power to revitalize artisanal production, threatened by big industry. Donald E. Thomas places Rudolf Diesel and the early history of the diesel engine in this context, and his book should appeal to those interested in understanding the relationships between science, technology, industry and society.

The history of the diesel engine provides a model of technological change through interaction between invention, development and innovation. In order to decentralize industry by providing a source of power for artisans, Rudolf Diesel translated concepts in thermo-

dynamics into an idea (invention) of a hot-air engine, based on the Carnot cycle. Trained at the Technical University (Technische Hochschule) in Munich, Diesel stressed the importance of thermal efficiency and, like leading academic experts, did not realize the difficulties of building an engine. Diesel found industrial backing but had to modify his theory of isothermal combustion using a large ratio of air to fuel in order to develop a working engine (development). Thomas analyses the invention and developmental stages and notes throughout the methods Diesel used to protect his original idea, embodied in his patent of 1892. Modifying the theory undermined the patent, which Diesel's enemies duly noted.

Successful innovation, or making the engine marketable, did not follow smoothly either. Diesel, and representatives from his industrial backers — Augsburg Engine Works and Krupp Works — prematurely announced a successful engine in 1897. Diesel suffered a breakdown and was unable to participate in overcoming the problems which became evident after diesel engines were placed in factory environments. As a result Diesel left the innovation stage to the Augsburg Engine Works, which helped form MAN in 1898. During the decade before his apparent suicide in 1913, Diesel faced financial problems and was little involved in engineering work on his engine.

Thomas accepts the widely held notion among German historians of a feudalized German middle class and combines it with the stages of invention, development and innovation used in the history of technology. Thus, he makes an important contribution to both fields by showing how engineers promoted technological change to preserve a social order threatened by that change. This is a finely crafted biography that goes beyond the usual limits of the genre. □

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# L'enfant du paradis

Benno Müller-Hill

**La Statue Intérieure.** By François Jacob. Seuil/Éditions Odile Jacob, Paris: 1987. Pp.357. FF98.

FRANÇOIS Jacob is one of the great molecular biologists of our time. The Jacob-Monod theory of operon control is a milestone in the understanding of living systems; the paper describing it appeared in *Journal of Molecular Biology* in 1961, and was the starting point of many careers, my own included. When a man of Jacob's standing writes an autobiography, others can turn to it in the hope of finding the hidden springs of scientific productivity. *La Statue Intérieure*, however, is far more than the story of a voyage of discovery in science.

Jacob surprises the reader: he devotes the first third of his book to his childhood. And what a happy childhood it seems to have been. A loving and charming mother, adored by her son, and a respected father. A French-Jewish general as grandfather, who told his grandchild a few days before his death, "There is no God. You and your children are my only hope". A grandmother who braved the Gestapo by demanding to be respected as the widow of a general. And an uncle who bought various coloured inks to encourage the young François to enjoy writing. Then came the terror of school, and the first encounter with the bully shouting "dirty Jew" (François fought back); the last visit to the synagogue; and his first great love amidst the magnificent landscape of Normandy.

In 1936 Jacob began to study medicine. But with the German invasion of France, his world collapsed. The French upper class and the generals feared the Communists more than the Nazis, and within a few days France had capitulated. Jacob drove with three friends to the south west, and with one of them decided to join de Gaulle's Free French movement. They found a ship and left for Britain in the nick of time.

In England his military training was not in the artillery as he would have liked, following in his grandfather's footsteps, but as an army doctor. (In a revealing passage he tells of walking through London in his new uniform, and being accosted by a French whore; he joins her, and out of politeness accepts the money she gives him out of patriotism.) From England he was posted to Chad, in central Africa, where a small contingent of French soldiers under the command of de Gaulle was advancing through the desert towards the Mediterranean. Here an event occurred that was to haunt him for years. He was sent to bring medical help to

an outpost and, in the moonlight, came without warning upon an armed German soldier. Jacob pretended not to have seen him and continued walking. Why, in his recurring nightmare, did not the soldier use his submachine gun?

After the German defeat in Africa, Jacob was shipped back to England, and on to Normandy. At a critical moment he chose to stay with a wounded friend instead of saving himself. The friend died, and Jacob himself was severely wounded. He arrived in Paris in an ambulance, when he had expected to enter the city in triumph. It was weeks until his relatives found him.

For Jacob the early years after the war were awful. Many of the brothers in arms who had left France to fight against the Nazis had died, and his Jewish friends had disappeared without trace. His first love had become engaged to somebody else. Back at medical school, he found that many of the professors and students had continued normal university life during the war and now had the advantage; he was not only an invalid, but had also lost five years. In 1947 he finally got his medical degree but remained a lonely, lost man. He did some odd jobs, and worked for an unsuccessful pharmaceutical firm. He married, but more for the sake of having children than for love. And all the approaches of Communists, de Gaullists and Israelis failed to interest him. Their cause was not his cause.

Despite his lack of a scientific background, Jacob's ambition was to do research. The question was where? More or less by accident he went to the Pasteur Institute and asked André Lwoff for a job. Lwoff declined, not once but twice. Jacob tried for a third time, and his persistence was rewarded. At the age of 30 he began to learn about the biology of bacteriophage. And so, in the final third of his book, he turns to science.

Jacob gives an account of the pleasure of seeing the first clear plaque and recognizing it was a mutant. He describes how Elie Wollman had the idea of doing the interrupted mating experiment, on which they collaborated; Sol Spiegelman's setback in proposing an incorrect model for adaptation; and Leo Szillard's success in proposing a correct model without prior evidence. And then the breathtaking moment, when he went to the cinema with his wife, closed his eyes to shut out the boring scenes and suddenly realized that the key phenomena of phage lambda and the *lac* systems could be explained by a single theory. He told his wife — who failed to understand — and had no one else to share the revelation with. Lwoff and Monod were on holiday. When he was finally able to tell Monod, Monod was at first uninterested. But when they discussed it again the next day Monod caught fire with enthusiasm, proposing different

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*Jacob — lust for new ideas and experiments.*

phenotypes for operator and repressor mutants. Jacob realized that he already had an operator mutant in lambda, and then spent a month with Sydney Brenner at Caltech to isolate mRNA. Nothing worked until Brenner finally realized that they had omitted the magnesium from the experimental brew. Back they went to the laboratory, and to success.

A common pattern emerges from the book. Those who did excellent science were also personally courageous, many of them having risked their lives during the war. After that, science became not a career but the best possible way of living. What I liked about the book is that it makes perfectly clear who thought what and who did what. It reminds us that ideas have fathers, for all the intellectual promiscuity of modern times, and that two minds may be necessary in order to bring an idea to fruition. The book ends most appropriately with the author walking through the empty Luxembourg Gardens while thinking up another experiment.

*La Statue Intérieure* is a joy to read. It is soon to be published in English\*, but I hope that it will be translated into many other languages and so have the widest possible audience. It will guide students to science and explain science to the layman. Although edged with darkness, it is filled with a transcendent love of life and lust for new ideas and experiments. And for those who read French, it is a wonderful exercise to read Jacob's elegant prose and a work that is literary as much as it is scientific. □

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\* To be published in the United States next February by Basic Books.



# Perturbing events in the Galaxy

Gerry Gilmore

**The Galaxy and the Solar System.** Edited by Roman Smoluchowski, John N. Bahcall and Mildred S. Matthews. *University of Arizona Press: 1986. Pp.483. \$29.95.*

FEW, if any, dinosaurs will read this review. It is also well established that the biological extinction at the Cretaceous — Tertiary boundary about 65 Myr ago coincided with a period of increased impact cratering on the Earth. Presumably these phenomena are related, though the cause and effect relationship between a comet hitting Earth and the extinction of species is not yet clear. Nevertheless, astronomical events apparently have a direct effect on biological evolution on Earth. It is of obvious (self-) interest to understand why large comets occasionally hit the Earth, and to predict when the next such event will occur. One clue to this came with the announcement in 1984 of a periodicity of about 30 Myr in the record of biological extinctions. Such a periodicity and its consequences dominate this collection of review articles, despite the somewhat misleadingly general title.

The current model for the origin of comets is that they are perturbed from a large cloud, about 100,000 times the diameter of the Earth's orbit, which contains about  $10^{11}$  comets in its outer parts and perhaps  $10^{13}$  in its dense inner core. These comets are stirred gravitationally by stars and giant clouds of interstellar molecules passing at random, and some of them fall close to the Sun (and to us). So was the dinosaurs' fate due to a random event? Not if such extinctions are periodic.

Periodicity of extinctions requires periodic gravitational perturbation of the comet cloud, which can be accounted for only by the orbit of the Sun in the Galaxy, or by the orbit of a companion planet or star round the Sun. The prospect of a solar companion — soon named 'Nemesis', or the 'Death Star' — controlling the fate of life on Earth led, in 1984, to a burst of interest in the popular press, and a rash of speculation on the matter.

Now that the dust has settled a little, Roman Smoluchowski and his colleagues have put together a well-presented collection of reviews giving the data, the models and their predictions, as well as several good accounts of the properties of the interstellar medium near the Sun. It is now clear that the variations in both the galactic tide and in the probability that the Solar System will pass very near a massive cloud in the interstellar medium, as the

Sun orbits around in the Galaxy, are too small to affect significantly the number of comets perturbed towards the Earth by interstellar clouds or by the variable galactic tide. So could a solar companion — planet or star — cause a comet shower of sufficient intensity to guarantee a large impact on the Earth? Given the low probability of such an impact, several billion comets would be required. One or more per day would cross the Earth's orbit, providing an entertaining spectacle at least.

A tenth planet seems unable to account for this. Reasonably detailed dynamical modelling shows that the comet showers it caused would be too long-lasting and of too low contrast to the background rate. Thus we are left with Nemesis. Apparently this could do the job, but only if it were allowed extremely contrived properties, not least among which is the instability of its orbit. If the Sun has a companion, it is just about to lose it for ever. The dinosaurs were indeed unlucky to live when they did.

There is, too, the question of periodicity in the record of biological extinctions. New statistical analyses and re-

investigation of the precision of the stratigraphical dating have now been completed. There are well-established and reliably dated impact events and biological extinctions at  $\sim 35$  Myr and  $\sim 65$  Myr ago, so that cometary impacts do indeed seem to cause mass extinctions. But earlier events are less well-dated, and the claimed periodicity, on which the validity of the companion-star model relied, is not statistically significant. In consequence of this, the exhaustive and repetitive discussion of the relevant arguments in this volume becomes of mostly historical interest. While it makes fascinating reading, the book is more appropriate for a long journey than as a text or reference work.

All in all there is little evidence that the Sun has a companion 'Death Star'. But it does seem likely that the dinosaurs were treated to a spectacular warning of their impending demise as several billion comets arrived — even if these were, as seems most likely, a consequence of the random passage of a now-distant star. □

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## Better reception

Gordon L.E. Koch

**Fundamentals of Receptor Molecular Biology.** By Donald F.H. Wallach. *Dekker: 1987. Pp.398. North America \$85, elsewhere \$102.*

ALTHOUGH one might not agree with all his pronouncements, one has to admit that Amstrad boss Alan Sugar's CTC (cut the crap) philosophy has made the products of the microelectronics revolution more widely accessible. So it is encouraging that similar ideas appear to be taking a hold in several areas of scientific publishing.

The CTC way of doing things is evident in the increasing use of a format in which large and complex subjects are broken down into bite-sized sections, each of which can be encapsulated in a single definitive statement. The experimental basis of the statement is then presented, together with an extensive bibliography to satisfy the demands of the more committed reader. The great merit of this approach is that the book can be read at several levels, or, perhaps more significantly, its scope assessed at a glance. It is particularly useful when dealing with subjects that require the coalescence of a variety of separate but interrelated disciplines under a single umbrella, such as molecular biology; indeed, the outstanding example of the success of this approach in recent times must be *Molecular Biology of the Cell*.

Wallach's stated intention in this book was to bring together accounts of a variety of disciplines bearing on the interactions between cells, especially at the molecular level. The bite-sized approach was therefore particularly appropriate, and an added concession to CTC is the use of a simple and, presumably, inexpensive print. It is this that has probably allowed the author to include some very contemporary studies on receptors. In general terms the book is a very satisfactory description of the key areas relating to receptor function. A particularly impressive feature is the extensive bibliography, which is a useful starting point for those wishing to delve into such specialist areas as hormone action, neurotransmitters and immune defence.

The general format of the book is as follows: it introduces the concept of information transfer between cells, examines the involvement of the cellular genetics systems in responses to information transfer, moves on to the role of the plasma membrane and then examines a number of systems such as the hypothalamus, pituitary, thyroid, pancreas, GI tract, steroid hormones, neurotransmitters, phagocytosis and the immune system in some detail, from the special perspective of intercellular information transfer. I thoroughly enjoyed reading *Fundamentals of Receptor Molecular Biology*, and learned much about subjects with which I thought I was quite familiar. □

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# Observed beaming of terrestrial myriametric radiation

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*Observations by the Dynamics Explorer 1 satellite provide validation of the theory that terrestrial myriametric radiation is produced by the linear conversion of electrostatic upper hybrid waves to electromagnetic radiation via a radio window. This theory predicts that the myriametric radiation is beamed relative to the magnetic field lines.*

TERRESTRIAL myriametric radiation<sup>1</sup> (TMR), also previously referred to as escaping continuum radiation<sup>2</sup>, consists of radio waves of wavelength approximately  $10^4$  m generated predominantly at the plasmopause<sup>3</sup> and magnetopause<sup>4</sup>. At frequencies greater than the magnetosheath plasma frequency ( $>30$  kHz) TMR propagates away into the interplanetary medium, but at lower frequencies it is trapped in the low density region between the plasmasphere and magnetosheath<sup>5</sup> where, after many reflections, it forms a continuum within the magnetospheric cavity. There is strong evidence that the source of TMR lies in electrostatic upper hybrid waves<sup>1-3,6</sup>, and numerous theories, both linear<sup>1,7</sup> and nonlinear<sup>8-11</sup>, have been proposed for the conversion from electrostatic to electromagnetic waves. One linear theory suggests that the conversion depends on the propagation of upper hybrid waves in density gradients at the plasmopause and magnetopause<sup>1</sup>. If the density gradient is normal to the magnetic field direction, upper hybrid waves can be transformed into electromagnetic Z-mode radiation<sup>12</sup> which is linearly converted to ordinary (O) mode radiation through a radio window<sup>13</sup>. A consequence of this conversion is that the O mode TMR propagates away from the source in two beams at angles with respect to the source magnetic field direction that are dependent on the ratio of electron cyclotron frequency to electron plasma frequency at the window location<sup>1</sup>. Results are presented here of TMR observed with the Dynamics Explorer 1 satellite (DE-1) that for the first time clearly show these two beams. These results demonstrate that the linear Z-mode to O-mode conversion mechanism is operating. A remote sensing technique<sup>14</sup> based on the theory is used to investigate the location and characteristics of the source region. Finally, the location of the TMR source region is demonstrated by direct measurement.

Figure 1 is a frequency-time spectrogram which shows electric field intensities from the plasma wave instrument (PWI) on DE-1. During this interval, the PWI was connected to a 200-m electric dipole antenna located in the spin plane of the spacecraft<sup>15</sup>. The ordinate shows the frequency scale (logarithmic) and the abscissa shows the Universal Time (UT), the magnetic latitude  $\lambda$ , the radial distance ( $R_E$ ), and the  $L$ -value of DE-1. The local electron cyclotron frequency ( $f_{ce}$ ) is plotted directly on the spectrogram. At frequencies above  $f_{ce}$ , and in a very limited latitude range near the magnetic equatorial plane, emissions are seen between the lower harmonics of  $f_{ce}$ . These are identified as  $(n + 1/2)f_{ce}$  electrostatic electron-cyclotron harmonic emissions<sup>16</sup>. The most intense member of this family lies at a higher frequency,  $f \approx 62.5$  kHz, which is believed to be the upper hybrid resonance frequency  $f_{UHR} = (f_{ce}^2 + f_{pe}^2)^{1/2}$ , where  $f_{pe} = 9N_e^{1/2}$  Hz is the electron plasma frequency and  $N_e$  is the electron concentration in  $m^{-3}$ . When DE-1 crossed the equatorial plane,  $f_{ce}$  was 7.6 kHz which is much less than  $f_{UHR}$ ; thus,  $f_{UHR} \approx f_p$ . This condition would appear to be true for most of the orbit shown. Hence the relatively weak band of noise seen in Fig. 1 in the range 60–100 kHz after 10:30 UT is identified as nonthermal continuum radiation whose lower frequency limit

lies near to the value of  $f_{pe}$  along the satellite trajectory<sup>5</sup>. After  $\sim 11:45$  UT, terrestrial kilometric radiation<sup>17</sup> (TKR) can be seen at frequencies above 100 kHz which has propagated to low latitudes from the auroral zones. This radiation tends to be more sporadic and intense than the TMR.

The radiation of interest is seen as two distinct 'blocks' near 100 kHz embedded in the nonthermal continuum on either side of the intense upper hybrid emission recorded at the equatorial crossing, and at a slightly higher frequency. They are seen to be nearly identical and separated by a zone of virtually no emission (except continuum) near the equatorial plane. They are symmetric, but not mirror images, about the equatorial plane and as will be shown shortly are propagating in the directions predicted by the linear Z-mode to O-mode conversion theory<sup>1</sup>. It should also be noted that there is a single block of emission straddling the upper hybrid event right at the magnetic equator which may also be TMR, the satellite in this case possibly being so close to the source that the two beams overlap. This emission is considered in more detail below.

## Theory

The linear window conversion theory has been dealt with elsewhere<sup>1,4,13,14,18,19</sup>. It requires that the plasma density gradient  $\nabla N_e$  in the source region be nearly perpendicular to the magnetic field vector  $\mathbf{B}_0$ . A result of the conversion is that the O mode electromagnetic radiation is beamed into the low density region in a plane containing  $\nabla N_e$  and  $\mathbf{B}_0$  at two angles,  $\beta = \pm \arctan(f_{pe}/f_{ce})^{1/2}$ , measured with respect to  $\mathbf{B}_0$ . If the intense upper hybrid waves are confined to the Magnetic Equator, as they seem to be in this case, the TMR would be beamed with respect to the magnetic equatorial plane at angles of  $\alpha = \pm \arctan(f_{ce}/f_{pe})^{1/2}$ , one beam to the north and the other to the south.

Thus, if conversion were to occur, the two TMR beams from the intense upper hybrid emission at 62.5 kHz near  $\lambda \approx 0^\circ$  in Fig. 1 should make angles of  $\pm \arctan(7.6/62.5)^{1/2} = \pm 19^\circ$  with respect to the equatorial plane. Since DE-1 actually passed through the source region for this frequency it was not in a position to observe those particular beams. Their observation would require another satellite in the magnetospheric cavity beyond DE-1 (see Fig. 2).

The two beams of TMR at frequencies above  $f_{UHR}$  in Fig. 1 presumably have their sources deeper in the plasmasphere, where the plasma density and hence the corresponding  $f_{pe}$  and  $f_{UHR}$ , are larger than 62.5 kHz. The decrease of plasma density as DE-1 moved along its trajectory is indicated by the drop in the lower frequency cutoff of the continuum radiation just after 10:30 UT.

Figure 2 shows the magnetic meridian plane trajectory of DE-1 for the period of time represented in Fig. 1. Marked on the trajectory is the point where the intense upper hybrid emission at 62.5 kHz was observed, and the two beams of 62.5 kHz TMR, assuming linear conversion, are shown emanating



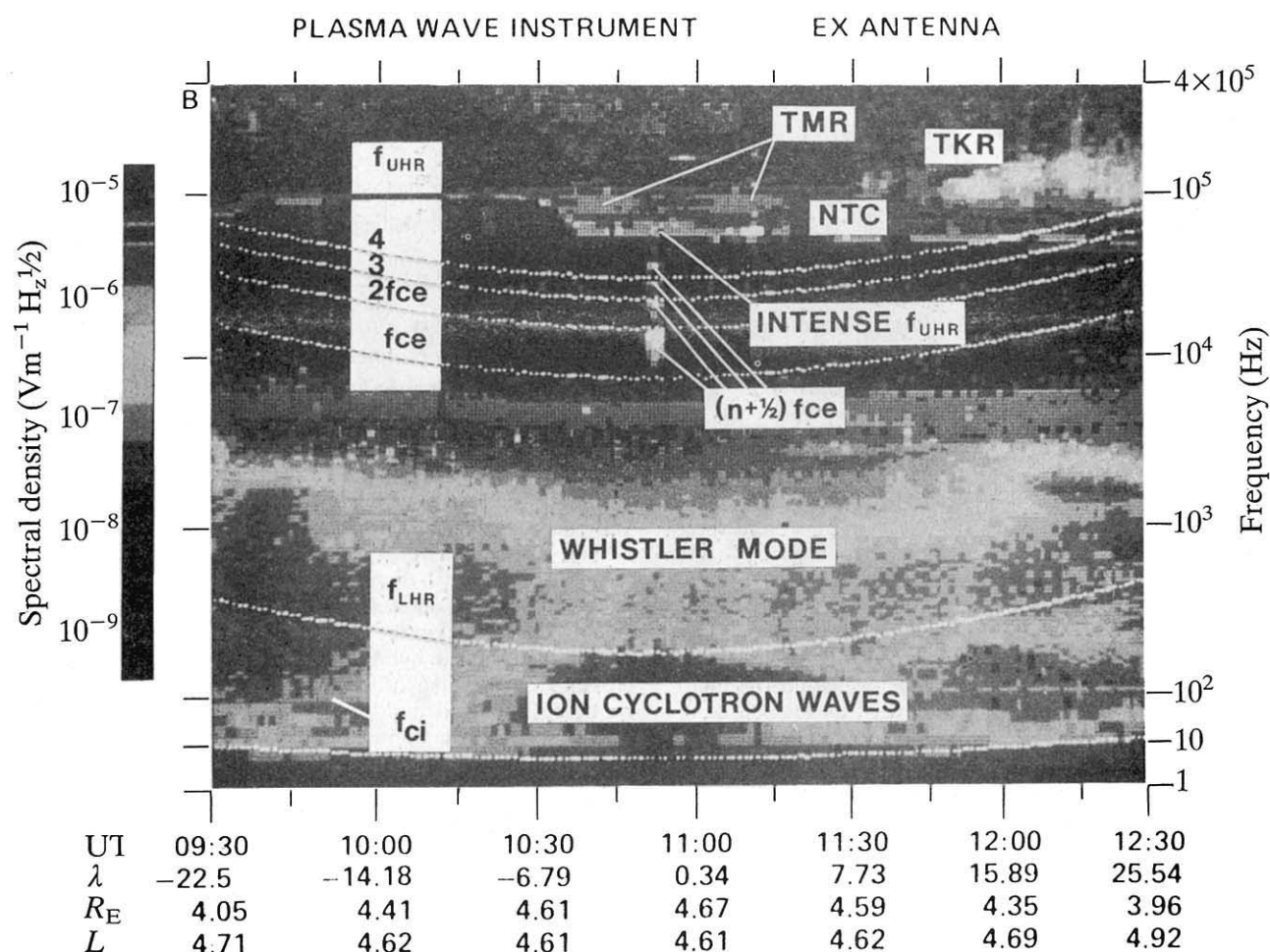


Fig. 1 A spectrogram obtained from the plasma wave instrument on the Dynamics Explorer 1 spacecraft during a south to north pass through the Magnetic Equator.

from it. The location of the source of the upper hybrid emission is slightly south of the Magnetic Equator obtained from the DE-1 magnetic field model. On the basis of this and other evidence<sup>20</sup>, we believe that the upper hybrid emission marks the correct location of the actual magnetic equator. Such discrepancies are easily within the range of uncertainty of existing magnetic field models at this radial distance. The magnetic field line labelled  $L = 4.665$  shows the field line that would occur if the magnetic equator is adjusted to coincide with location of the upper hybrid emission. As can be seen the satellite trajectory lies virtually along this magnetic field line, especially between 09:30 and 11:30 UT. Thus one would expect this part of the trajectory to be an equipotential and, since it is so close to the Magnetic Equator, to exhibit little variation of the plasma density. However, from the cut-off frequency of the continuum there is clearly a precipitous drop in plasma frequency from 94 kHz ( $N_e \approx 1.1 \times 10^8 \text{ m}^{-3}$ ) at 10:27 UT to 58 kHz ( $N_e \approx 4.2 \times 10^7 \text{ m}^{-3}$ ) at 10:37 UT. Although the satellite is also moving gradually in longitude, there is unquestionably a very steep density gradient at this time and location. The gradient is not detected north of the equatorial plane presumably because the satellite trajectory remained outside the plasmopause. This is probably one reason why the distribution of continuum and TKR in Fig. 1 is also not symmetrical about the Equator.

## Remote sensing

The relationship  $\beta = \pm \arctan(f_{pe}/f_{ce})^{1/2}$  allows one to demonstrate that the two beams of TMR in Fig. 1 were most probably produced by the linear Z-mode to O-mode conversion mechanism. A form of remote sensing can be used to determine the

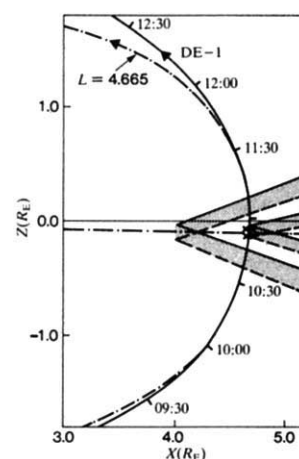
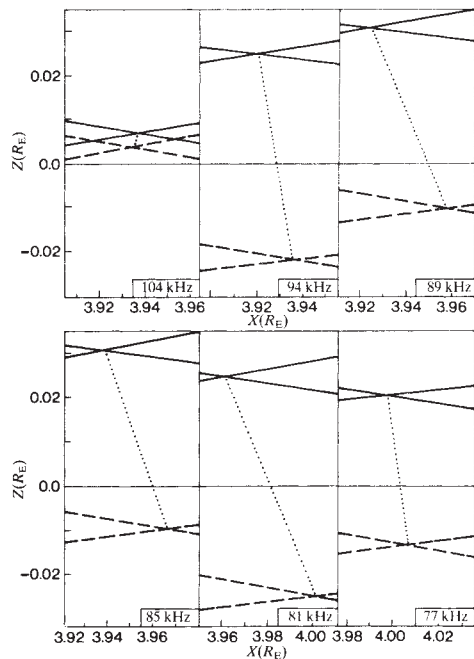
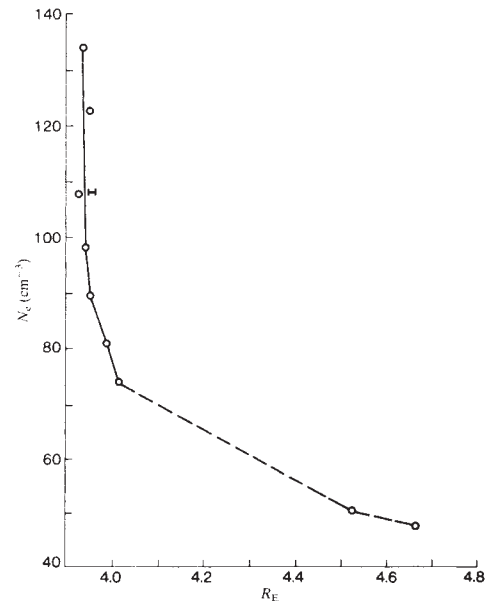


Fig. 2 The trajectory of DE-1 (curved full line) during the time when the data in the spectrogram in Fig. 1 were recorded. The model and corrected magnetic equators (full and chained lines respectively) are shown. The chained line labelled  $L = 4.665$  represents a magnetic field model that has been adjusted such that the field line passes through the UHR emission at the Magnetic Equator. Hypothetical sources and beams of TMR are shown. The outer beam pair is presumed to emanate from the 62.5 kHz UHR emission at  $4.665 R_E$ . Observation of these beams would require another satellite outside the trajectory of DE-1. The inner beam pair is assumed to be at a frequency of 100 kHz and to have its source at  $4 R_E$ . In both cases the sources have a small latitudinal extent about the Magnetic Equator.



**Fig. 3** Source location (dotted lines) of the TMR at 77 kHz to 104 kHz obtained by remote sensing. Negative sloping broken and full lines are possible source positions of the southern beam, whereas the positive sloping broken and full lines are corresponding source locations for the northern beam.



**Fig. 4** An approximate plasma density profile (full line) deduced from Fig. 3. The dashed line connects the profile to the points obtained from the TMR identified at 64 kHz (see Fig. 5) and the UHR emissions observed at  $4.665 R_E$ . The bar at  $N_e = 109 \text{ cm}^{-3}$  shows the magnitude of the error in the source position as estimated from Fig. 3.

position of the TMR sources at the different frequencies. This technique has been described in detail elsewhere<sup>14</sup> and only a brief outline will be given here.

Given that TMR of frequency  $f$  is emitted where  $f = f_{pe}$  and that it is beamed with respect to the source magnetic field at an angle which depends on  $f_{pe}$  and  $f_{ce}$ , one may determine, for a given magnetic field model, where an observer has to be located in order to receive the radiation. Conversely, knowing the magnetic coordinates of the observer one can determine the possible positions of the sources in a magnetic field model. For the Earth, it may be assumed that the magnetic field is dipolar within about  $4 R_E$ . If one concentrates on possible sources in the vicinity of the equatorial plane, the location and dimension of the TMR source in Fig. 1 can be determined. It is assumed here that propagation is in the magnetic meridian plane. Consider, for example in Fig. 2, a source of 100 kHz having a magnetic field-aligned latitudinal extent of  $2^\circ$  centred on the equatorial plane and located at  $4 R_E$ . The cyclotron frequency is assumed to be 12 kHz. Thus  $\beta \approx 70^\circ$  and the two beams should appear as shown in the figure. As DE-1 moves northwards towards the equator it observes the southern beam, first encountering the radiation from the southern edge of the source and then encountering the radiation from the northern edge of the source. As the satellite continues northwards past the Equator and into the Northern Hemisphere, it observes the northern beam, again receiving first the radiation from the southern edge of the source and then the radiation from the northern edge. If the source is not exactly uniform with latitude it is clear that the satellite would observe, not a mirror image about the equatorial plane, but a sequential repetition of the non-uniformities as it progresses northwards through the two beams. This repetition is clearly visible for the two blocks of TMR appearing in Fig. 1.

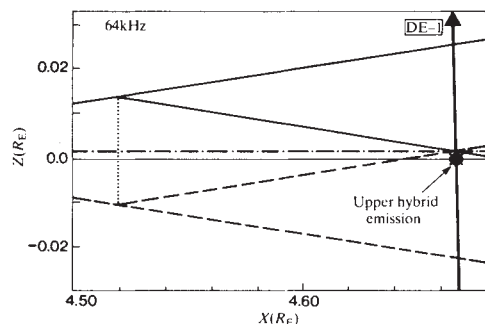
The remote sensing technique was applied to the beam edges at different frequencies in Fig. 1 to investigate the source positions and dimensions, and the results are presented in Fig. 3 which shows the source locations of TMR for six frequencies ranging from 77 kHz to 104 kHz. The abscissae  $X$  represent the radial distance in the equatorial plane and the ordinates  $Z$  the

distance normal to that plane. Each panel has a pair of dashed lines and a pair of full lines. The lower, negatively-sloping dashed lines represents possible source locations for the lower edge of the southern beam, whereas the upper positively-sloping dashed lines give the possible source positions for the lower edge of the northern beam. As was stated earlier, these beam edges should have originated from the same part of the source, namely its southern edge. Therefore where the two dashed lines intersect should yield the position of the southern edge of the source, whereas where the two similarly-deduced full lines intersect should give the location of the northern edge of the source. Thus the apparent sources determined by this remote sensing technique for the different frequencies are represented by the dotted lines connecting these two intersections. Clearly there is a considerable variation in their dimensions extending from only  $\sim 0.0025 R_E$  (16 km) at 104 kHz to  $\sim 0.05 R_E$  (320 km) at 81 kHz. The sources are not magnetic field-aligned normal to the equatorial plane, and this is most probably due to inaccuracies in determining precisely the Magnetic Equator or the beam edges, or to temporal and spatial variations of the plasmopause during the 15-min interval between the measurements. It may also be that the density gradient is not exactly radial during the time period involved, so that the beams are not emitted strictly in the magnetic meridian.

For each of the frequencies in Fig. 3 the mean source location can be used to obtain an approximate plasma density profile of the plasmopause, since the theory states that  $f = f_p = 9(N_e)^{1/2}$ . The result is shown in Fig. 4 which clearly implies a very steep density gradient between  $3.93 R_E$  and  $4 R_E$  which would be conducive to mode conversion according to the linear theory. Although this density gradient cannot be associated directly with that suggested from the sharp decrease in  $f_{pe}$  at 10:27 UT and  $L \approx 4.665$ , it is compatible with the existence further in of a very steep plasmopause at this time.

Attention was drawn earlier to the emission, tentatively identified as TMR, near to and straddling the upper hybrid waves at the magnetic equator. The result of remote-sensing using the two edges of this emission at  $f = 64 \text{ kHz}$  is shown in Fig. 5. The



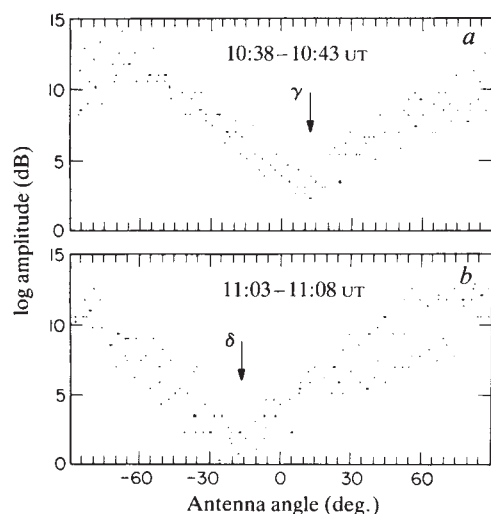


**Fig. 5** Remote sensing of the edges of the continuous 'block' of TMR at 64 kHz. The dashed lines represent the beams from the southern source edge and the full lines the beams from the northern edge. A source at radial distance less than  $4.52R_E$  would have resulted on the observation of two distinct beams.

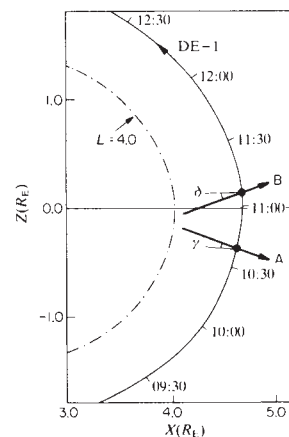
trajectory of DE-1 and the position of the 62.5 kHz upper hybrid emission (at the corrected Magnetic Equator) are illustrated. The possible source positions of the 64 kHz TMR lie on the negatively-sloping dashed line (southern edge of possible source) and positively-sloping full line (northern edge of possible source). If it is assumed that the source is normal to the Magnetic Equator, then the minimum radial distance of the source would be  $4.52R_E$ . Source locations at a smaller radial distance would result in DE-1 observing two separate beams with a zone of no emission in between. This source location and corresponding plasma density were included in Fig. 4, along with the density determined from the upper hybrid emission at  $4.665R_E$ . They are joined to the other points by dashed lines to emphasize that the  $4.52R_E$  source at 64 kHz is only an inner limit, assuming that the emission was actually TMR. A possible reason for the absence of TMR at frequencies between 64 kHz and 77 kHz may be that the density gradient is not sufficiently large for the conversion mechanism to operate efficiently.

### Measured wave directions

The intensity of the TMR in Fig. 1 is, before smoothing, found to be strongly modulated by the rotation of DE-1's electric



**Fig. 6** Amplitudes of the TMR signals in Fig. 1 as a function of the DE-1 antenna angle, measured with respect to nadir at the times and frequencies indicated. The relatively deep nulls at angles  $\gamma$  and  $\delta$  give the wave ray directions shown plotted in Fig. 7.



**Fig. 7** The wave directions deduced from Fig. 6, along with the DE-1 orbit and the  $L = 4$  L-shell from which the emissions seem to have originated.

dipole antenna. For compact sources near the spin plane of the antenna, the modulation pattern, as a function of the antenna rotation angle, should be characterized by distinct nulls from which the wave direction can be determined<sup>21</sup>. The DE-1 spacecraft spins in a 'reverse-cartwheel' mode, with its spin axis oriented perpendicular to the orbit plane. Because of this orientation, and also because the satellite's orbit plane lies nearly in the magnetic meridian, the geometry is ideal for spin-null direction-finding measurements, thereby providing us with an opportunity to test the linear conversion model by making direct measurements.

Figure 6 shows the measured electric field intensities as a function of the antenna angle with respect to the spacecraft-Earth line (nadir direction) projected into the spin plane, with panels *a* and *b* pertaining to the two blocks of TMR discussed above. In both cases pronounced spin nulls were clearly evident. The angles  $\gamma$  and  $\delta$  presumably correspond to antenna alignment with the propagation direction, since the wave electric field in this case should be almost perpendicular to the wave direction. The two angles  $\gamma$  and  $\delta$ , on opposite sides of the spacecraft nadir before and after the apparent equatorial crossing, thus measure directly the wave directions for the two components of TMR.

Figure 7, in the same format as Fig. 2, shows these wave directions at the locations where they were observed. As predicted, the two directions were approximately symmetric on opposite sides of the emission axis and in excellent agreement with those deduced from the linear conversion model. Moreover, the apparent source location, near  $L = 4$  and 85 kHz, is also consistent with the plasmopause position deduced from the remote sensing technique described in the previous section. The theoretical model, therefore, is not only internally consistent with the detection of TMR beams where they were observed, but also directly confirmed by the spin-null direction-finding measurements.

### Conclusions

The observations presented here strongly support the picture of TMR beaming which is predicted by the linear Z-mode to O-mode conversion theory. Taken with the evidence that the conversion mechanism also seems to be operative in the magnetospheres of Jupiter<sup>22</sup> and Saturn<sup>23</sup>, it would appear that the process may also be of importance in a wider, astrophysical context.

Investigation of this and other similar events is continuing. In particular, the possibility that the emissions derive from a non-radial density gradient is under study. Also the theory predicts that the southern TMR beam should be right-hand

polarized, whereas the polarisation of the northern beam should be left-handed. Although the PWI can sometimes measure wave polarization directly, the TMR emissions in this case were too weak for a reliable polarization analysis. We are currently searching for more intense TMR events, for which a polarization analysis is possible, and the results of such polarization measure-

ments and other studies will be reported separately.

We thank R. C. Olsen for bringing this event to our attention and for supplying the original (colour) version of the spectrogram in Fig. 1 (see ref. 24). The research at the University of Iowa was supported by NASA and by the Office of Naval Research.

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# Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells

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*A detailed analysis of the residues within an immunogenic peptide that endow it with the capacity to interact with Ia and to be recognized by T cells is presented. Ia interacts with only a few of the peptide residues and overall exhibits a very broad specificity. Some residues appear to interact both with Ia and with T cells, leading to a model in which a peptide antigen is 'sandwiched' between Ia and the T-cell receptor.*

IN general, T helper cells do not recognize native protein antigen, but only antigen that has been physically altered (denatured or partially degraded) and subsequently displayed in association with major histocompatibility complex (MHC) class II(Ia) molecules by antigen presenting cells (APC)<sup>1</sup>. It has been suggested that the final step in the antigen-specific, MHC-restricted activation of T cells is the creation of a trimolecular complex consisting of Ia, 'processed antigen' and the T-cell receptor<sup>2</sup>. Until recently the affinities and specificities involved in the creation of this complex were unresolved and the subject of considerable controversy. In the past two years however, it has been shown that purified Ia molecules can bind immunogenic peptides<sup>3,4</sup> with a  $K_D$  of  $\sim 5 \mu M$ . We have subsequently found that the ability of a given Ia molecule to serve as a restriction element for the presentation of an antigen correlated with the capacity of that Ia to bind the antigen. Furthermore, data obtained using several unrelated immunogenic peptides restricted by the same Ia suggested that each Ia possesses a single peptide binding site of broad specificity<sup>5</sup>.

This paper uses the presentation of the immunogenic peptide derived from chicken ovalbumin, Ova 323-339, by the Ia molecule, I-A<sup>d</sup>, as a model system for a detailed analysis of the structural requirements for peptide-I-A<sup>d</sup> interaction, and for T-cell activation. We have previously identified Ova 323-339 as being immunodominant in H-2<sup>d</sup> mice<sup>6</sup>, and have shown that this peptide binds to I-A<sup>d</sup>, but not to I-E<sup>d</sup>, an Ia molecule which will not present this peptide<sup>7</sup>. In this study we examined a large series of analogues of Ova 323-339 and tested their capacity to bind to I-A<sup>d</sup> and to stimulate two Ova 323-339/I-A<sup>d</sup>-specific T-cell hybridomas. By the use of N- and C-terminal truncated

peptides we defined a core region of seven amino acids which is required for peptide/I-A<sup>d</sup> interaction. This core region shows a significant degree of structural similarity with corresponding regions of three other unrelated peptides which, like Ova 323-339, bind well to I-A<sup>d</sup>. Using a series of analogues with single amino-acid substitutions at the 11 positions 325-335, the same Ia-interacting core region was identified. By this analysis only four residues were involved in binding to Ia. The majority of the analogues showed binding to Ia comparable to that of the parent peptide, illustrating the very conservative nature of antigen-Ia interaction. In contrast, examining the capacity of two T-cell hybridomas to recognize the analogue series, the T cells were found to be much less permissive than Ia. Residues corresponding to 9 of the 11 positions substituted were involved in recognition by one or the other T-cell hybrid. Ia and T-cell receptor interacting residues were intermingled, and some residues apparently interacted with both Ia and T-cell receptor. To explain these results, we propose a model for the trimolecular complex in which the peptide is 'sandwiched' in a  $\beta$ -sheet or extended conformation between Ia and the T-cell receptor.

## Region required for Ia interaction

To define which residues within Ova 323-339 were involved in the interaction with Ia, we made a series of peptides that were sequentially truncated from the N-terminal or C-terminal end of the peptide. The capacity of these peptides to bind to I-A<sup>d</sup> was measured by their capacity to inhibit the binding of the full-length radiolabelled peptide, [<sup>125</sup>I]-labelled Ova 323-339, to I-A<sup>d</sup>. The data are shown in Table 1. Deletions from the C-terminal end were without appreciable effect until the seventh



**Table 1** Effect of N- and C-terminal truncations of Ova 323–339 on I-A<sup>d</sup> binding capacity

| Ova peptide | Sequence               |   |   |   |   |   |   |   |   |   |   |   |   |   | Relative I-A <sup>d</sup> binding capacity* |   |   |        |
|-------------|------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---------------------------------------------|---|---|--------|
|             | C-terminal truncations |   |   |   |   |   |   |   |   |   |   |   |   |   |                                             |   |   |        |
| 323-339     | I                      | S | Q | A | V | H | A | A | H | A | E | I | N | E | A                                           | G | R |        |
| 323-336     | -                      | - | - | - | - | - | - | - | - | - | - | - | - | - | E                                           |   |   | 1.1    |
| 323-335     | -                      | - | - | - | - | - | - | - | - | - | - | - | - | N |                                             |   |   | 1.0    |
| 323-333     | -                      | - | - | - | - | - | - | - | - | - | - | E |   |   |                                             |   |   | 0.6    |
| 323-332     | -                      | - | - | - | - | - | - | - | - | A |   |   |   |   |                                             |   |   | 0.2    |
| 323-331     | -                      | - | - | - | - | - | - | H |   |   |   |   |   |   |                                             |   |   | <0.005 |
|             | N-terminal truncations |   |   |   |   |   |   |   |   |   |   |   |   |   |                                             |   |   |        |
| 325-339     | Q                      | - | - | - | - | - | - | - | - | - | - | - | - | - | -                                           | - | - | 1.1    |
| 326-339     |                        | A | - | - | - | - | - | - | - | - | - | - | - | - | -                                           | - | - | 0.5    |
| 327-339     |                        |   | V | - | - | - | - | - | - | - | - | - | - | - | -                                           | - | - | 0.3    |
| 328-339     |                        |   |   | H | - | - | - | - | - | - | - | - | - | - | -                                           | - | - | 0.014  |
| 329-339     |                        |   |   |   | A | - | - | - | - | - | - | - | - | - | -                                           | - | - | <0.005 |

\* Peptide antigens were synthesized, cleaved and HPLC purified. I-A<sup>d</sup> molecules were obtained by affinity purification of lysates of B-cell lymphoma A20 with the monoclonal anti-I-A<sup>d</sup> antibody MKD6 (ref. 7). The I-A<sup>d</sup> interacting capacity of each peptide is expressed as the amount of unlabelled Ova 323–339 divided by the amount of truncated peptide needed to inhibit the binding of <sup>125</sup>I-radiolabelled Ova 323–339 to I-A<sup>d</sup> by 50% as measured by a gel filtration method<sup>4</sup>. The data represent the averages of 2–4 experiments.

residues, E<sub>333</sub>, was removed. This deletion decreased the inhibitory capacity relative to the full length peptide by threefold. Further removal of A<sub>332</sub> resulted in complete loss of binding activity. At the N-terminal end, a slight decrease in binding activity was associated with the removal of Q<sub>325</sub> and A<sub>326</sub>, with a marked decrease in binding activity being associated with removal of V<sub>327</sub>, and a complete loss of binding activity being associated with removal of H<sub>328</sub>. Thus, the critical residues required for interaction with I-A<sup>d</sup> were contained within the sequence VHAAHA.

### Single amino acid substitutions

To further characterize the amino-acid positions that were critical for binding of the peptide to I-A<sup>d</sup>, we synthesized a series of analogues of the 14-residue peptide, Ova 323–336. This peptide is highly stimulatory for certain T cells and is also fully active in the I-A<sup>d</sup> binding assay. To study the functional role of each residue within the peptide, five amino-acid substitutions (two conservative, one semi-conservative, and two non-conservative) were made for each of the 11 residues. These 55 analogue peptides were then compared for their binding capacity to I-A<sup>d</sup> relative to the full length peptide Ova 323–339.

The data from these experiments are summarized in Fig. 1a. First, it is apparent that most of the substitutions introduced into the peptide had little effect on the binding capacity of the peptide for I-A<sup>d</sup>. Only 9 of the 55 substitutions had a significant deleterious effect on binding activity ( $P < 0.01$ ) and only 7 substitutions led to more than a fivefold decrease in binding capacity. The most dramatic decrease in binding involved 5 of the substitutions at residues V<sub>327</sub> and A<sub>332</sub>; the two non-conservative substitutions at each of these two positions led to a 10–85-fold reduction in binding activity. The substitution of a glycine for A<sub>330</sub> or A<sub>332</sub> also resulted in a tenfold decrease in binding activity. As glycine is known to have a strong influence on the secondary structure of peptides<sup>8</sup> and as this was the only substitution at A<sub>330</sub> that resulted in a decreased binding activity, we propose that A<sub>330</sub> is not itself involved as a contact residue in the interaction with I-A<sup>d</sup>, but rather, it is likely that the glycine substitution altered the capacity of the contact residues to interact with I-A<sup>d</sup>. Less drastic effects on the I-A<sup>d</sup> binding capacity of non-conservative substitutions were also observed at residues H<sub>328</sub> (fivefold decrease) and E<sub>333</sub> (fourfold decrease).

The degree of inhibition observed with this series of single amino-acid substitutions confirmed and extended the data obtained with the truncated peptides shown in Table 1. Thus, the most dramatic effects were obtained upon deletion or substi-

tution of residues V<sub>327</sub> and A<sub>332</sub>, and both methods also implicated, albeit at a less important level, H<sub>328</sub> and E<sub>333</sub>. It is of interest that one of the substitutions, Y for E<sub>333</sub>, significantly enhanced the binding activity of the peptide ( $P < 0.01$ ). Although we have interpreted the effects on binding to Ia of truncations and single amino-acid substitutions as being caused by alterations or deletions of contact residues, we cannot exclude the possibility that some of the observed effects are indirect and due to alterations in peptide conformation.

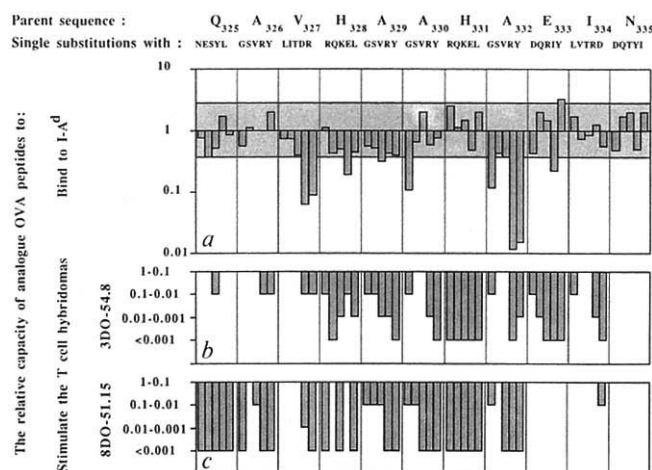
### Structural similarity

We have identified 3 other peptides from unrelated proteins that also bind strongly to I-A<sup>d</sup> (ref. 5 and unpublished observations). Because each of these peptides competitively inhibit the binding of Ova 323–339, it is most likely that each of these peptides bind to the same site on I-A<sup>d</sup> as the Ova peptide, and therefore, they should share the critical structural features required for such binding. To estimate the structural relationship between two aligned peptide sequences, we adapted the method described by Grantham<sup>9</sup> and Padlan<sup>10</sup>. A dissimilarity value for each pair of corresponding amino acids is obtained from a table that takes into account differences in atomic composition, polarity and molecular volume. The dissimilarity values are then normalized for mean dissimilarities of random substitutions and

**Table 2** Determination of 'best fit' alignment between Ova 327–333 and Ha 130–142

| Ova 'master' sequence | V | H | A | A | H | A | E | ASD  |
|-----------------------|---|---|---|---|---|---|---|------|
| Ha 130–142 Alignment  | H | N | T | N | G | V | T | 45.0 |
|                       | N | T | N | G | V | T | A | 48.3 |
|                       | T | N | G | V | T | A | A | 34.1 |
|                       | N | G | V | T | A | A | C | 45.0 |
|                       | G | V | T | A | A | C | S | 44.6 |
|                       | V | T | A | A | C | S | H | 25.5 |
|                       | T | A | A | C | S | H | E | 38.6 |

To determine the best fit alignments of the sequence corresponding to residues 130–142 of influenza haemagglutinin (Ha 130–142) with residues 327–333 of Ova, all the possible alignments were generated by sliding the test sequence (Ha 130–142) along the master sequence (Ova 327–333). For each alignment, average structural dissimilarity (ASD) values were then calculated as described by Padlan<sup>10</sup>. The particular alignment yielding the lowest ASD value (underlined) was selected as the best fit alignment of the two sequences.



**Fig. 1** I-A<sup>d</sup>-interacting capacity and T-cell stimulatory activity for hybridomas 3DO-54.8 and 8DO-51.15 of single substitution analogues of Ova 323-336. Single-substitution analogues of Ova 323-336 were synthesized by combining automated peptide synthesis<sup>7</sup> with the method described by Houghten<sup>15</sup>. Five different substitutions (two conservative, one semi-conservative, two non-conservative) were introduced into each of the eleven positions 325-335, yielding a total of 55 single-substitution analogues. These peptides were then tested for I-A<sup>d</sup> binding (a), by the method described in Table 1. Each bar represents the arithmetic mean of two to four independent determinations. Shaded area represents 99% confidence limits. The same set of single-substitution analogues was also tested for stimulatory activity on 3DO-54.8 (b) and 8DO-51.15 (c). T cells ( $2 \times 10^5$ ) were incubated in 0.3 ml medium together with various antigen concentrations, ranging from 0.1 to 100  $\mu\text{g ml}^{-1}$  and  $10^5$  A20 cells that served as antigen-presenting cells. After 24 h the IL-2 concentration in each culture supernatant was determined in the IL-2 dependent HT-2 cell assay<sup>17</sup>, and the amount of each peptide, relative to the amount of the unsubstituted Ova 323-336, needed to induce the production of 80 U  $\text{ml}^{-1}$  of IL-2 was measured. Each peptide was then scored in one of four classes of reactivity. Each bar represents the average of four to eight independent determinations.

the average (average structural dissimilarity; ASD) is calculated for each peptide alignment. As the experiments with truncated peptides and peptides with single amino-acid differences indicated that the sequence of Ova 327-333 (VHAHAHE) encompassed most of the I-A<sup>d</sup> binding activity we used this 7-residue peptide as a 'master' sequence for comparison with the 3 unrelated peptide antigens that have thus far been identified as also binding strongly to I-A<sup>d</sup>. For each of the 3 peptides we calculated the ASD values of all the possible linear alignments to Ova 327-333. An example of such an analysis is shown in Table 2, where the sequence of influenza haemagglutinin (Ha) 130-142 is compared to the sequence of Ova 327-333. For one of the alignments the ASD value was considerably lower (25.5) than for all the other alignments. This alignment was selected as the 'best fit'. Best-fit alignments were similarly selected for the two other I-A<sup>d</sup>-interacting peptides derived from sperm-whale myoglobin (Myo) 106-118, and staphylococcal nuclease (Nase) 101-120 (Table 3). Although the overall homology between this set of four peptides is not overly impressive, a common structural motif can be discerned. Position 1 is the most highly conserved being V or I in the 4 peptides; position 2 contains the basic amino acids, H or R, or the polar residue T; positions 3 and 4 have a strong tendency to be non-polar; position 5 is not highly conserved containing basic or acidic residues as well as non-polar residues. This residue has been identified as the most important residue for T-cell recognition (ref. 11 and Fig. 1b,c) and thus may not be expected to be conserved in these different

**Table 3** 'Best fit' alignments of unrelated peptides with good I-A<sup>d</sup> binding capacity

|      |         | Residue number |   |   |   |   |   |   |
|------|---------|----------------|---|---|---|---|---|---|
|      |         | 1              | 2 | 3 | 4 | 5 | 6 | 7 |
| Ova  | 327-333 | V              | H | A | A | H | A | E |
| Ha   | 135-141 | V              | T | A | A | C | S | H |
| Myo  | 112-118 | I              | H | V | L | H | S | R |
| Nase | 104-110 | V              | R | Q | G | L | A | K |

Best fit alignments between Ova 327-333 and sequences of three unrelated peptides, which are good I-A<sup>d</sup> binders were generated as described in Table 2.

peptides; position 6 is conserved and contains residues with short ( $\text{CH}_3$  or  $\text{CH}_2\text{OH}$ ) side chains. Position 7 contains charged residues, either basic or acidic.

When we compared the VHAHAHE sequence with the sequence of 13 peptides that failed to bind to I-A<sup>d</sup> (ref. 5 and unpublished observations), we detected a significantly higher ASD value than those obtained for the good binders. The mean of the ASD values for the best fit alignments of the good binders was  $26.5 \pm 0.6$  (s.e.m.) and that of the non-binders was  $31.7 \pm 1.3$  ( $P < 0.0025$  by a one-tailed *t* test). Supportive of the validity of these alignments, we have been able to identify a peptide that shared the motif found in the good I-A<sup>d</sup> binders by scanning the amino-acid sequence of sperm-whale myoglobin. This peptide, Myo 66-72, has the sequence VTLTAL. When a peptide containing this core region, Myo 63-78, was tested for binding to I-A<sup>d</sup>, it was found to be as good a binder as the other four peptides in this category (50% inhibition at 11  $\mu\text{M}$ ). Also we have recently localized the I-A<sup>d</sup> binding region of Ha 130-142 and Myo 106-118 by studying a series of truncated peptides. These analysis indicate that the core regions predicted on the basis of the best fit alignments are correct (unpublished observations).

The homologies shared between this set of good binders to I-A<sup>d</sup> further confirm the conclusions that were made based on the analysis of the analogue peptides of Ova; namely that the requirements for binding to I-A<sup>d</sup> are highly permissive and that a large number of variations on a common structural theme allow for good binding. It is noteworthy that this type of specificity is predicted by the determinant selection hypothesis which requires that a few Ia molecules have the capacity to bind a large universe of 'processed' foreign antigens.

## T cell recognition

Three patterns of T-cell recognition have been defined by the reactivity of a large panel of T-cell hybridomas reactive with truncated versions of the Ova 323-339 peptide. For all three types of T cells an intact core region 327-333 was required for T-cell stimulation. In addition, residues that extended on both sides of the core region were required. Two of the fine specificity patterns were typified by the hybridomas 8DO-51.15 and 3DO-54.8. For 8DO-51.15, five residues at the N-terminal side were critical and any N-terminal deletion led to a complete loss of stimulatory activity. In contrast, the reactivity of 3DO-54.8 was not as critically dependent on an intact N-terminal region as the first 2-3 residues could be deleted without significant loss of stimulatory activity but required more residues beyond the core region at the C-terminal end. Thus, there are clear differences in the fine specificity for each of the hybrids but all required recognition of residues that lie outside the core region of the Ova peptide.

To define the requirement for T-cell recognition of the Ova peptide further, the same set of peptide analogues of Ova 323-336 that were used to define the I-A<sup>d</sup> interaction site were also used to analyse the T-cell recognition requirements of these two T-cell hybrids. The data are summarized in Fig. 1b,c. The first



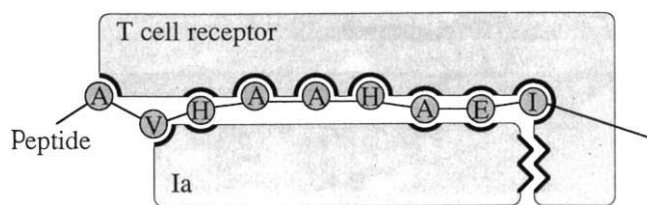
**Table 4** Summary of involvements of each Ova 325-335 amino-acid residue

| Position<br>in Ova<br>sequence | Residue | I-A <sup>d</sup><br>interaction | T-cell recognition |           |
|--------------------------------|---------|---------------------------------|--------------------|-----------|
|                                |         |                                 | 3DO-54.8           | 8DO-51.15 |
| 325                            | Q       | -                               | -                  | ++        |
| 326                            | A       | -                               | -                  | +         |
| 327                            | V       | ++                              | -                  | -         |
| 328                            | H       | +                               | ++                 | +         |
| 329                            | A       | -                               | ++                 | ++        |
| 329                            | A       | -                               | ++                 | ++        |
| 330                            | A       | ?                               | +                  | ++        |
| 331                            | H       | -                               | ++                 | ++        |
| 332                            | A       | ++                              | -                  | +         |
| 333                            | E       | +                               | ++                 | -         |
| 334                            | I       | -                               | +                  | -         |
| 335                            | N       | -                               | -                  | -         |

point that is evident from these data is that T-cell recognition is far more sensitive to single amino-acid substitutions than is I-A<sup>d</sup> interaction. For 3DO-54.8, 62% of the substitutions led to a greater than tenfold decrease in antigenic activity and 24% led to a greater than thousandfold decrease in activity. For 8DO-51.15, 62% of the substitutions resulted in a greater than tenfold decrease in activity and 45% resulted in a greater than thousandfold decrease in antigenicity. In a similar approach Geysen<sup>12</sup> used single-substituted analogues to examine peptide antigen-antibody interactions. The results obtained in the antibody system were similar to the results obtained here in the T-cell system. Thus, the specificity of T-cell receptor recognition of antigen-MHC complexes appears to be equivalent to the specificity of antibody recognition.

When our data on the effects of the substitutions at individual positions are scrutinized, a complicated pattern emerges. For 3DO-54.8, at some positions, the five substitutions had little or no effect on T-cell recognition (Q<sub>325</sub>, A<sub>326</sub>, N<sub>335</sub>) or an effect compatible with the effect of the substitution on I-A<sup>d</sup> binding (V<sub>327</sub>, A<sub>332</sub>). At other positions the requirement for the native residue was absolute and any substitution, conservative or non-conservative led to a marked decrease in reactivity (H<sub>328</sub>, A<sub>329</sub>, H<sub>331</sub>, E<sub>333</sub>). At yet other positions only some, usually conservative, substitutions were permissible whereas other substitutions led to a marked decrease in activity (A<sub>330</sub>, I<sub>334</sub>). A similar analysis of 8DO-51.15 indicates that residues E<sub>333</sub>, I<sub>334</sub> and N<sub>335</sub> were either not involved or minimally involved in T-cell recognition; V<sub>327</sub> involvement was accounted for by the effects of the substitutions on I-A<sup>d</sup> binding; Q<sub>325</sub>, A<sub>329</sub>, A<sub>330</sub> and H<sub>331</sub> were critical residues that could not tolerate any substitutions; and substitutions at residues A<sub>326</sub>, H<sub>328</sub> and A<sub>332</sub>, were tolerated in some, but not other, instances.

Of particular interest is the effect of substitutions at the four residues that had been identified as being involved in the interaction with I-A<sup>d</sup>: V<sub>327</sub>, H<sub>328</sub>, A<sub>332</sub> and E<sub>333</sub>. The loss of activity in T-cell recognition associated with substitutions at V<sub>327</sub> could be readily accounted for by the effect on I-A<sup>d</sup> binding since the same substitutions that caused a decrease in I-A<sup>d</sup> binding (D and R) were the same residues involved in decreased T-cell stimulatory capacity for both T-cell hybrids. In contrast, the only substitution for H<sub>328</sub> that affected binding to I-A<sup>d</sup> was H → E, whereas all of the substitutions decreased the stimulatory capacity of the peptide for 3DO-54.8 and three of the four substitutions other than E decreased the stimulatory activity for 8DO-51.15. The only substitution for E<sub>333</sub> that affected I-A<sup>d</sup> binding was E → I, whereas all five substitutions markedly decreased the stimulatory capacity for 3DO-54.8. Similarly, when A<sub>332</sub> was substituted by G, R or Y, the binding activity for I-A<sup>d</sup> was decreased. These same substitutions led to a decreased stimulatory activity for 3DO-54.8. But the stimulatory



**Fig. 2** Model of the interaction between the Ova peptide, Ia, and the T-cell receptor. Accented concavities in the Ia and T-cell receptor indicate putative contact sites with antigen. The peptide is sandwiched in an extended conformation between I-A<sup>d</sup> and the T-cell receptor to allow shared recognition of some residues (H<sub>328</sub>, A<sub>332</sub>, E<sub>333</sub>). All the other residues are shown as either pure T-cell or pure Ia-interacting residues. The T-cell recognition of Ia residues is also indicated.

activity of the analogues for 8DO-51.15 was also markedly decreased by substitution of V for A, a substitution that had no effect on I-A<sup>d</sup> binding, nor on stimulation of 3DO-54.8. Thus we obtained evidence that three of the four residues identified as being involved in the binding to I-A<sup>d</sup> were also involved in T-cell receptor recognition as substitutions that did not alter I-A<sup>d</sup> binding did alter T-cell recognition of the peptide.

The second major finding from these data is that, with the possible exception of V<sub>327</sub>, for which the effects of substitutions could be explained on the basis of the effects on I-A<sup>d</sup> binding, and N<sub>335</sub> for which there were no effects on any of the substitutions, all the other residues were implicated as being involved in T-cell receptor interaction for one or the other of the two T-cell hybrids studied. For each of these remaining nine residues, at least one of the substitutions led to a greater than thousandfold decrease in stimulatory activity. That the effect of substitutions was mediated by a failure of a particular residue to interact with a T-cell receptor rather than being mediated by some gross alteration of the conformation of the peptide, was indicated by the finding that with the exception of the substitutions at A<sub>329</sub> and H<sub>331</sub>, at least one of the substitutions for each of the other residues that resulted in a drastic decrease (>thousandfold) in T-cell stimulation of one of the hybrids, had either no effect or a much less drastic effect (10-100-fold) on the stimulation of the other hybrid.

## Conclusions

The effects of single amino-acid substitutions on the binding to I-A<sup>d</sup> and on recognition by T cells are summarized in Table 4. Two major conclusions can be made. First, only two residues were strongly implicated in the binding to I-A<sup>d</sup> with two others being involved to a lesser extent. Only non-conservative substitutions at these four positions had a marked effect on I-A<sup>d</sup> binding activity. The effect of the A to G substitution at position 330 as discussed above, is most readily explained by postulating an effect on peptide conformation, rather than by suggesting that this residue is a fifth I-A<sup>d</sup> contact residue. Collectively, these data would indicate that a great many peptides should have the capacity to bind to I-A<sup>d</sup>, a suggestion that is in keeping with the analysis of the structural homology between the unrelated peptides that are capable of binding I-A<sup>d</sup> with relatively high affinity as well as in keeping with the determinant selection hypothesis.

The second conclusion relates to the conformation that the peptide assumes when it is bound to I-A<sup>d</sup> in the form of an immunogenic complex. It has been suggested that immunogenic peptides form  $\alpha$ -helical structures that segregate the amino acids of the peptide such that one surface of the  $\alpha$ -helix is involved in Ia interaction while the other surface is involved in T-cell receptor interaction<sup>13,14</sup>. The data obtained in this study with

Ova 323–339 does not appear to be consistent with such a model. First, when the peptide is modelled into an  $\alpha$ -helix the residues involved in Ia interaction do not segregate from the residues involved in T-cell receptor recognition. The lack of segregation is also evident from a consideration of the finding that nine of the eleven residues examined were implicated as being involved in T-cell receptor interaction. Second, as detailed above, evidence was obtained that three of the residues involved in Ia interaction were also involved in T-cell recognition. This is clearly inconsistent with the  $\alpha$ -helical model as proposed. Rather, the data suggest a model in which the peptide assumes a two-dimensional planar conformation such that each of the residues of the peptide would be in a position to interact both with Ia and the T-cell receptor; thus the peptide is 'sandwiched' between Ia and T-cell receptor in an extended coil or  $\beta$ -sheet conformation (Fig. 2). Preliminary data obtained with peptides

that consist of the core region 327–333 which has been extended by 'nonsense' residues with particular secondary structure preferences<sup>8</sup> suggest that a  $\beta$ -sheet is the most likely conformation assumed by the Ova peptide in its interaction with I-A<sup>d</sup>.

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**Note added in proof:** Using an entirely different method to analyse T-cell epitopes, Rothbard<sup>17</sup> has identified the HAAH portion of the Ova peptide and the HVLH region of Myo 106–118 as representing structures common to T-cell antigens. The generic structure he has identified is: charged (or glycine), hydrophobic, hydrophobic, polar (or glycine). All four core regions shown in Table 3 contain this type of sequence.

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## LETTERS TO NATURE

### The discovery of a millisecond pulsar in the globular cluster M28

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**Here we report the discovery of a pulsar with a 3 millisecond period in the core of the globular cluster M28. The existence of a millisecond pulsar in such a cluster, where there are frequent interactions between cluster stars, provides strong confirmation of the theory that the high rotation rate derives from accretion in a binary system. The pulsar is now isolated, having no binary companion.**

Following the 1982 discovery<sup>1</sup> of the millisecond pulsar, PSR1937+21, a number of authors<sup>2,3</sup> proposed a new class of rapidly rotating pulsars, spun-up by accretion of material from a binary companion at some stage in its evolution<sup>4</sup>. Consequently, millisecond pulsar progenitors should be strong X-ray emitters typical of the low mass X-ray binary (LMXB) systems found in densely populated globular clusters, where formation of binary systems is thought to be particularly favourable.

Motivated by this prediction, Hamilton, Helfand and Becker<sup>5</sup> undertook a point source survey with the Very Large Array (VLA) at 1,465 MHz of 12 nearby globular clusters. Only a single cluster, M28 (NGC6626), had a compact object within one core radius of its centre. This object was undetectable at the higher frequency of 5 GHz suggesting a rather steep spectral index, typical of pulsars.

Within a year, lower-resolution Clark Lake observations<sup>6</sup> at 31 and 57 MHz had established the existence of a decametre

wavelength source within the M28 field of view indicating a spectral index of  $\sim -2.4$ . VLA data<sup>7</sup> at 327 MHz subsequently enabled unambiguous identification of this source as the high-frequency compact object already known, confirming a spectral index of  $-2.44$ , which by itself was very strong evidence of an underlying pulsar. The measurement of  $(35 \pm 10)\%$  linear polarization in the 327-MHz data added further weight to this conclusion.

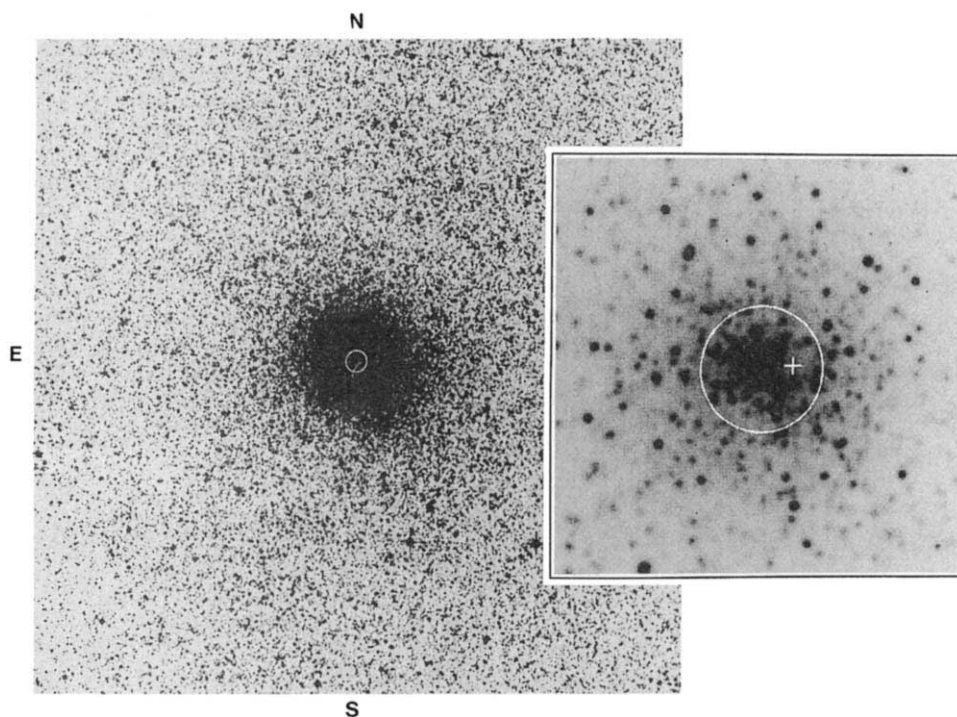
The globular cluster M28 had been searched unsuccessfully for pulses at Molonglo in the course of the 1977 survey<sup>8</sup> although it would have been detected had the period been greater than 40 ms. Searches for a shorter-period pulsar require a rapid data-sampling rate in many radio bands that are sufficiently narrow to avoid the effects of interstellar dispersion; the amount of data and the computing requirements vary roughly as the inverse square of the minimum detectable period. At Jodrell Bank we have assembled hardware dedicated to searches for millisecond pulsars, but until recently did not have a computer with a sufficiently large capacity to process the extended data sequences required to detect weak sources like that in M28.

Here we present the successful results of a new search using the 76-m radio telescope at Jodrell Bank, UK and a Cray-XMP computer at the Los Alamos National Laboratory, USA, and subsequent observations which have confirmed the existence of the pulsar.

The 76-m MK IA telescope was equipped with a cooled 1.4-GHz receiver sensitive to both right and left-handed circular polarization. Total power outputs from 32 contiguous 1-MHz filterbank channels were recorded every 300 microseconds and stored on magnetic tape for off-line analysis. To minimize the storage requirements one-bit digitization was employed. Although this results in a 20% reduction in sensitivity compared with multi-bit digitization, it is an acceptable compromise.

M28 was observed for 90 min on 6 December 1986 and again on 2 January 1987, each observation providing a data set of 16 million time samples from each of the 32 frequency channels. These data were separately dedispersed off-line for 84 values of dispersion measure lying between 20 and 300 pc cm<sup>-3</sup>; each of the 84 values provided a 16 million point time sequence for input to a fast Fourier transform periodicity search algorithm<sup>9</sup>.





**Fig. 1** The globular cluster M28 and the position of the millisecond pulsar. The main photograph shows a 21.5-arc min square centred on M28 (Royal Observatory, Edinburgh, UK Schmidt Telescope). Inset is a CCD image of the core obtained by Drs I. King and S. Djorgovski from the 1.5-m Cerro Tololo telescope with a Johnson B filter. In both diagrams, the white circle represents the extent of the core and is centred on the centroid of the cluster and has a radius of 21 arc s (ref. 11). The position of the pulsar is shown by a cross.

This entire process on the two data sets took five hours on the Cray-XMP supercomputer.

During the searches, the Fourier spectrum below 51 Hz was ignored, as were all harmonics of  $126.7 \pm 0.5$  Hz arising from the interference of an aviation radar station at Cleve Hill. The only statistically significant periodicity not apparently associated with the Cleve Hill radar was found in the 2 January 1987 data near 327.4 Hz, peaking at 27 times the mean power level at 327.40932 Hz for a dispersion measure of around  $140 \text{ pc cm}^{-3}$ . Harmonics near 654.8 Hz and 982.2 Hz were also detected.

The December data set was then searched for the equivalent  $327.40571 \pm 2$  Hz barycentric frequency, but displaced from the January result by about 70 wave numbers in the 16 million point Fourier transform owing to the change of the component of the Earth's velocity along the line of sight to M28. We found a peak at  $327.40574 \pm 3$  Hz with 8.5 times mean power at the same dispersion measure.

Subsequent observations of pulse arrival times at Jodrell Bank over the period from 2 June to 9 June 1987 have confirmed the existence of the pulsar<sup>10</sup>, designated PSR1821-24, and the values for the period, period derivative and dispersion measure are given in Table 1.

The position of the pulsar used to correct the arrival times to the solar system barycentre is given in Table 1 and is that measured with the VLA by Erickson *et al.*<sup>7</sup>. In each coordinate there is an estimated error of 1 arc s which gives rise to an additional error of  $10^{-12}$  s in the calculated period. The corresponding error in the period derivative is insignificant.

Figure 1 shows the globular cluster M28 and indicates the location of the pulsar within it. The exact pulsar position is shown on the inset CCD (charge-coupled device) photograph of the cluster core. The pulsar is seen to lie within the very core of the cluster as identified by Peterson and King<sup>11</sup>.

The pulse profile is displayed in Fig. 2 and shows a double pulse, with a separation of  $105 \pm 5^\circ$  (longitude) between the components. There appears to be significant radiation between the components and also following the second component. Substantial linear polarization is also present.

The location of a millisecond pulsar within a globular cluster provides compelling confirmation of the suggestion<sup>2,3</sup> that mil-

lisecond pulsars are a population of old pulsars which have been spun up by the accretion processes seen in low mass X-ray binary systems. It also provides an explanation for the absence of the binary companion from which material was transferred during spin-up. Two theories seem appropriate: first, a binary system, that may itself have been formed in a dissipative encounter, might undergo a sufficiently close interaction with a third, relatively swift, cluster star causing disruption of the binary

**Table 1** Parameters of the M28 pulsar PSR1821-24

|                        |                                              |
|------------------------|----------------------------------------------|
| Period                 | $3.05431449090 \pm 0.00000000004 \text{ ms}$ |
| Period derivative      | $< 10^{-18} \text{ ss}^{-1}$                 |
| Epoch                  | MJD 46952.0                                  |
| Dispersion measure     | $120 \pm 1 \text{ pc cm}^{-3}$               |
| Mean flux density      | $1.1 \pm 0.3 \text{ mJy}$                    |
| Right Ascension (1950) | 18 h 21 min 27.50 s                          |
| Declination (1950)     | $-24^\circ 53' 51.0''$                       |
| Distance               | 5.8 kpc                                      |
| Galactic longitude     | $7.8^\circ$                                  |
| Galactic latitude      | $-5.5^\circ$                                 |

system after the end of the X-ray phase. Alternatively, an almost head-on collision between an isolated neutron star and a non-compact field star, with subsequent tidal capture, would have naturally led to an LMXB phase, and may end in coalescence, resulting in a spun-up single pulsar<sup>12,13</sup>.

The pulsar lies at a distance  $z \sim 600 \text{ pc}$  below the galactic plane, so that the line of sight samples a region outside the main ionized layer intercepted by most pulsars. Using the Lyne, Manchester and Taylor<sup>14</sup> model for the galactic electron density distribution in the Galaxy, a dispersion measure of  $120 \text{ pc cm}^{-3}$  5.5 degrees below the plane corresponds to a distance of 3.9 kpc. Optical measurements on the other hand seem to prefer a value of 5.8 kpc for the cluster<sup>15</sup>. If this is correct, then the actual electron density along the line of sight is lower than in the model; probably because the density of the extended component does not increase towards the galactic centre as assumed by the Lyne, Manchester and Taylor model.

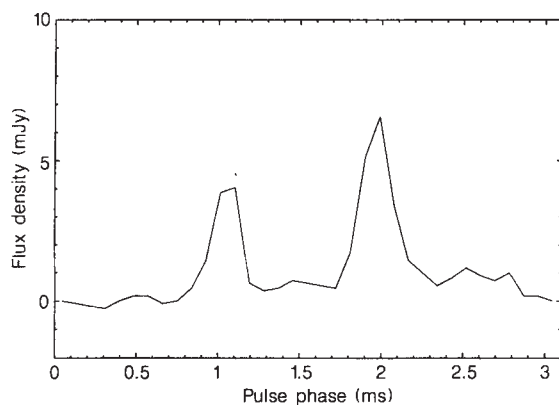


Fig. 2 The pulse profile obtained at 1,420 MHz. The total instrumental broadening amounts to about 130  $\mu$ s.

Although the pulsar appears relatively weak, the pulses are quite narrow so that during daily 4-h observations using only 8-MHz total bandwidth near 1,400 MHz, pulse arrival times can be measured with an accuracy of  $\sim 3 \mu$ s. Use of an appropriate dedispersing receiver of larger bandwidth should allow future measurements with  $\sim 1 \mu$ s accuracy. PSR1821-24 is located nearly one radian away from the other isolated millisecond pulsar PSR1937+21, so that timing measurements on both pulsars will be valuable in solving for Solar System parameter errors. Furthermore, millisecond pulsars may provide a more smoothly running time standard than offered by terrestrial standards such as the caesium beam clocks. It may now be possible to demonstrate this by regular observations of more than one pulsar over a period of several years. Blandford, Romani and Applegate<sup>16</sup> have warned that gravitational perturbations by the cluster stars may cause an anomalous period second derivative which will degrade the usefulness of this pulsar in searches for cosmological radiation. It may nonetheless still provide a valuable clock, as the timescale of the changes in the second derivative is expected to be several thousand years. Accurate timing measurements may also allow the determination of the proper motion of the cluster.

Already, with just one week of high-quality timing data on PSR1821-24, we have been able to place a useful upper limit on the period derivative and hence an upper limit on the surface magnetic field of  $2 \times 10^9$  gauss.

M28 lies at a distance of 5.8 kpc<sup>15</sup>; the pulsar is therefore intrinsically quite bright, even though the flux density of 25 mJy at 400 MHz is lower than for most detected pulsars. Evidently there must be a considerable population of pulsars in globular clusters, although the general searches at present under way may not be sufficiently sensitive to detect them.

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## The prodigious warp of NGC4013

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Non-planar distortions, or warps, in the outer gas layers of galaxies are more the rule than the exception, although the degree of warping may differ drastically from one galaxy to another<sup>1-3</sup>. H I radio synthesis observations reveal that the edge-on Sbc<sup>4</sup> galaxy NGC4013 has the largest regular H I warp so far observed. It extends to a large height above the plane of the galaxy, and begins abruptly at just the radius where photometry indicates the end of the luminous disk. Furthermore, at precisely this position, the rotational velocity is seen to drop by 25 km s<sup>-1</sup>. These observations can only be explained by a situation in which not only the disk-light distribution, but also the disk-mass distribution, suddenly approach zero at the radius of the warp onset. On the basis of a modelling of the H I gas distribution, we conclude that our line of sight to NGC4013 is, by coincidence, one which shows the warp most clearly.

NGC4013 was observed for 2  $\times$  12 hours with the Westerbork Synthesis Radio Telescope in June, 1982. The synthesized beam was  $14.0 \times 19.7$  arc s full width at half maximum (FWHM) with a velocity resolution of 33.2 km s<sup>-1</sup>. The total measured flux was 39.1 Jy km s<sup>-1</sup>, implying a total H I mass of  $1.1 \times 10^9$  solar masses. All channel maps were smoothed to a resolution of  $30 \times 30$  arc s to increase the signal-to-noise ratio, especially in the warped region. A distance of 12 Mpc has been assumed.

The smoothed total H I distribution is given in Fig. 1 and the result after integration of the data cube in the direction perpendicular to a position angle of 50° 'strip'  $l, v$ , or longitude-velocity diagram, made by integrating emission along the minor axis is shown in Fig. 2. This position angle is a good compromise between that of the outer H I distribution and the disk. It is clear from Figs 1 and 2 that the outer gas layers of NGC4013 are highly warped. Also, as can be seen from the  $l-v$  diagram, the rotational velocities on both sides of the galaxy are lower in the warped region than in the disk. To unravel the morphology and kinematics in an unambiguous way, it was necessary to model the gas layer of NGC4013.

The presently fashionable model for warped galaxies is the

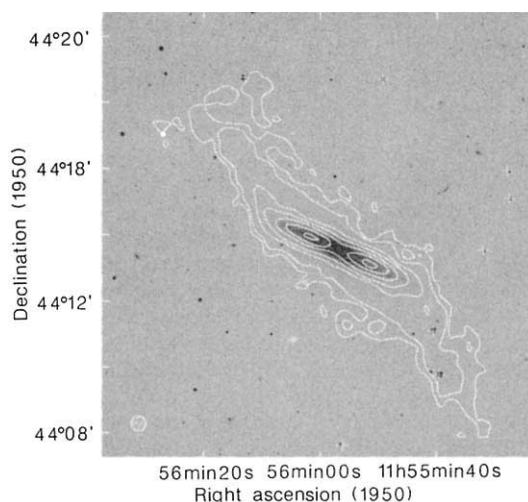
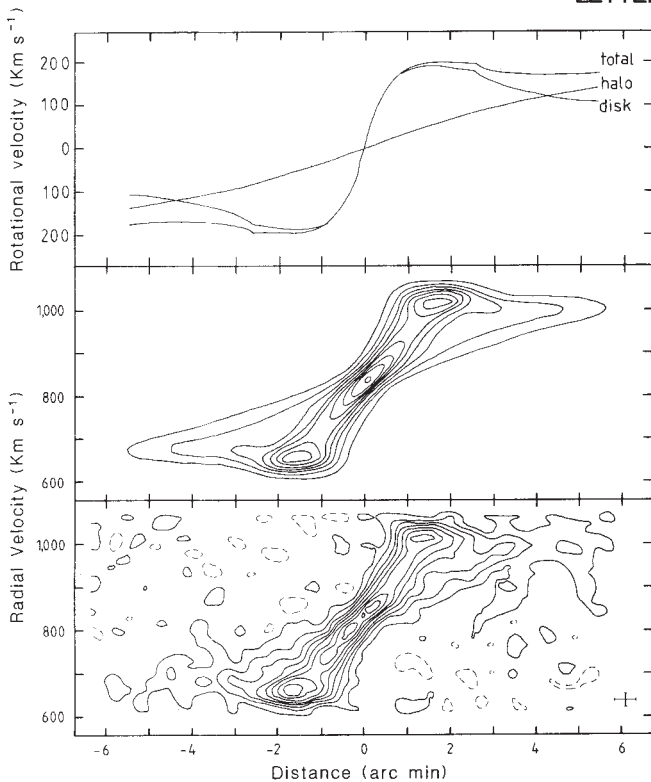


Fig. 1 The total H I distribution of NGC4013 superposed on an optical photograph in the F-band. The beamsize is  $30 \times 30$  arc s. Contour levels are at  $1.7 \times 10^{19}$ ,  $1.1 \times 10^{20}$ ,  $5.6 \times 10^{20}$ ,  $1.1 \times 10^{21}$ ,  $2 \times 10^{21}$ ,  $2.9 \times 10^{21}$  and  $3.8 \times 10^{21}$  H-atoms cm<sup>-2</sup>.



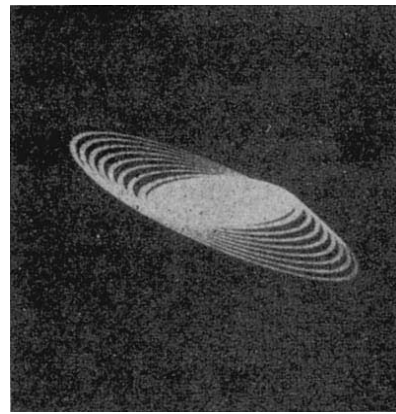


**Fig. 2** Bottom: Observed  $l$ - $v$  diagram. This diagram is obtained after integration perpendicular to a position angle of  $50^\circ$  (major axis position angle =  $66^\circ$ ) over the extent of the H I emission. This position angle was chosen to minimize the contribution of noise. Levels range from  $-8.4$  K to  $104$  K in steps of  $\sim 14$  K. Middle: Resulting  $l$ - $v$  diagram after modelling. Top: Rotation curves obtained after fitting a disk-halo mass model to the data. The 'total' rotation curve is used in the modelling.

so-called 'tilted ring model'<sup>1</sup>. The disk and warp are divided up into a number of concentric rings of differing orientations. We have used this model, but have restricted the warped rings to intersect the central disk along the same line (see Fig. 3). For the present exactly edge-on galaxy, the warp can then be described with three parameters: first, the 'warp angle'  $\alpha$ , which is the angle between the plane of the central disk and the line through the extrema (lying  $1/4$  circle from the line of intersection) of the concentric rings. In other words, if  $\alpha = 0$ , there is no warp. Second, the angle  $\psi$ , which is the angle between the line perpendicular to the line of intersection and the line of sight. For  $\psi = 90^\circ$ , the warp, whose maximum is then in the plane of the sky, shows up very clearly; for  $\psi = 0$ , it is probably only visible as a diffuse thick H I disk. The third parameter is, naturally, the radius at which the warp starts. One must, of course, also specify the surface density, thickness, rotational velocity and velocity dispersion of each ring.

To compare the ring model with our data, an exact simulation of the process of H I line observations was made. First, all the modelled line channels were calculated and smoothed in both of the two spatial directions and in velocity to match the resolution of the observations. Next, the smoothed channel set, or data cube, was integrated in velocity to get the total H I map, and then integrated in the direction perpendicular to the plane of the galaxy to obtain the  $l$ - $v$  diagram. Both the total H I map and  $l$ - $v$  diagram can be compared with the observations, and if the kinematic and structural parameters are chosen correctly, these should fit the data simultaneously.

Our first modelling effort was to try and match the observations by assuming a constant rotational velocity, and varying the angles  $\alpha$  and  $\psi$ . This turned out to be impossible for any choice of parameters. To 'lower' the observed velocities in the



**Fig. 3** Morphological description of the H I ring structure used in the model calculations.

outer region;  $\psi$  had to be so small that the model total H I map entirely failed to agree with the observations. Thus, there was no alternative other than to lower the rotational velocity by  $\sim 25$  km s<sup>-1</sup> for radii larger than  $165$  arc s.

A galaxy whose disk has an edge will show a sudden drop in the rotation curve<sup>5,6</sup>. However, a disk-like density distribution alone cannot produce the constant (lowered) rotational velocity observed in NGC 4013 beyond  $R_{\max}$ . A dark halo is needed to accomplish this. Therefore, we constructed a disk-halo model to fit the constant rotation of  $195$  km s<sup>-1</sup> for radii smaller than  $R_{\max}$ , and a rotational velocity of  $170$  km s<sup>-1</sup> for radii beyond  $R_{\max}$ . For the disk we assumed the following distributions<sup>6,7</sup>:

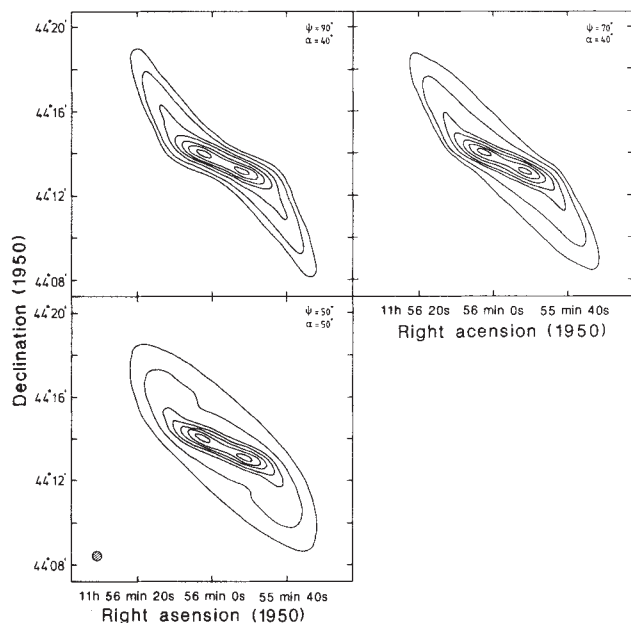
$$\rho = \rho_0 e^{-R/h} \text{sech}^2(z/z_0) \quad \text{for } R < R_{\max} \\ = 0 \quad \text{for } R > R_{\max} \quad (1)$$

For the halo, we have used an isothermal sphere. Adopting the maximum disk hypothesis<sup>8</sup> the observed rotation curve can be obtained for a total disk mass of  $5 \times 10^{10} M_\odot$ ,  $M_{\text{halo}}/M_{\text{disk}} = 0.3$  for  $R < R_{\max}$ , and a core radius for the halo of  $15$  kpc. This means that  $M_{\text{halo}}/M_{\text{disk}} = 1.8$  for  $R < 20$  kpc, the largest radius out to which the rotational velocity can be accurately derived. The corresponding rotation curve is plotted in Fig. 2, which was used as input for the channel map modelling.

In Fig. 4 we show the resulting total H I maps for three models with different values of  $\psi$ . For every model we have tried to optimize the agreement with the observations by adjusting  $\alpha$ . Examining Fig. 4 one can see that a value of  $\psi = 70^\circ$  might just fit the observations, but that  $\psi = 90^\circ$  is better. This means that we just happen to be situated along the right line of sight to see the warp so clearly. The  $l$ - $v$  diagrams corresponding to the three models differ only slightly, so we only show the result for  $\psi = 90^\circ$  in Fig. 2. An interesting point is that the warp starts precisely at the radius ( $9.5$  kpc) where the rotation curve drops and the stellar disk ends. For all simulations a constant thickness of  $1.3$  kpc width at half-maximum density (HPW) and a velocity dispersion of  $10$  km s<sup>-1</sup> were used for the hydrogen layer, but the conclusions are not sensitive to these assumptions.

The origin of warps is still not clearly established. One explanation is that the once regular H I disk has been distorted by tidal forces resulting from the interaction with another galaxy<sup>9</sup>. In the present instance, a good candidate for such a model would be NGC 3938, which has almost the same redshift as NGC 4013. The distance between the two galaxies is  $200$  kpc. Another possible explanation is that the warp is the result of a recent infall of gas from outside the galaxy.

A large warp cannot persist for more than a few rotation periods in the presence of a disk-like potential alone<sup>10</sup>. If a spherical or halo potential is added, the warp will last much longer. The very abrupt onset of the warp in this galaxy therefore indicates that at  $R = R_{\max}$  there is a sudden change from a



**Fig. 4** Resulting total H I maps for three models. The angles  $\alpha$  and  $\psi$  are described in the text. From comparison with the observed total H I, we conclude that  $\psi > 70^\circ$ .

disk-like to a more spherical potential. This is additionally supported by the fact that at this radius both the light and the rotational velocity suddenly drop.

It is instructive to compare this galaxy with galaxies of similar morphological type and dimensions to see if the large warp can be related to other galactic properties. In Table 1 we give parameters of NGC4013 together with those of the well-studied regular spiral NGC3198 and the edge-on galaxy NGC891.

The first three items in Table 1 might be related. For  $R_{\max}/h \sim 4$  a drop in the rotation curve should be measurable, but for  $R_{\max}/h \sim 5$  the drop has almost completely disappeared<sup>5</sup>. So

**Table 1** Comparison of galaxy parameters

| NGC4013                                                           | NGC3198                                                | NGC891                                                            |
|-------------------------------------------------------------------|--------------------------------------------------------|-------------------------------------------------------------------|
| Optical edge at $R/h = 4.1$                                       | Possible edge near $R/h \sim 5$                        | Optical edge at $R/h = 4.3$                                       |
| Drop in rot curve                                                 | Flat rot curve                                         | Possible drop in rot curve                                        |
| Warp                                                              | No warp                                                | No clear warp                                                     |
| $M_{\text{halo}}/M_{\text{disk}} = 0.3$ at $R = R_{\max} = 4.1 h$ | $M_{\text{halo}}/M_{\text{disk}} = 0.8$ at $R = 4.1 h$ | $M_{\text{halo}}/M_{\text{disk}} = 2.0$ at $R = R_{\max} = 4.3 h$ |
| $h = 2.3 \text{ kpc}$                                             | $h = 2.7 \text{ kpc}$                                  | $h = 4.9 \text{ kpc}$                                             |
| $V_{\max} = 195 \text{ km s}^{-1}$                                | $V_{\max} = 150 \text{ km s}^{-1}$                     | $V_{\max} = 225 \text{ km s}^{-1}$                                |

the difference in rotation curve between NGC4013 and NGC3198 (drop versus flat) can be related to the fact that the disk of NGC3198 is relatively larger. Additionally, a larger disk could be responsible for damping out the warp.

In NGC3198 there is no clear evidence for the 'truncation feature' in the rotation curve around  $R_{\max}$ , while there is some evidence for this in NGC891 on the south-west side<sup>11</sup>. The major difference between NGC4013 and the other two galaxies is in the halo-disk mass ratio within  $R_{\max}$ . For NGC4013 and NGC3198, these ratios have been derived from an analysis of the rotation curve using the maximum disk solution, and therefore the ratios are actually lower limits. For NGC891, the disk mass is estimated from the thickness of the H I layer, and is therefore a more direct measurement<sup>12</sup>. Eventually we will be able to use the observations presented here to make a similar

estimate for NGC4013 by employing the detailed data for the inner gaseous disk.

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## Observation of possible superconductivity at 230 K

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Recently, Chu<sup>1</sup> announced the observation of sharp drops of up to two orders of magnitude in the resistance of several Y-, Sc- and La-based multi-phase Ba-Cu oxide samples at temperatures as high as 240 K. The resistance drops reported did not survive the thermal cyclings for magnetic measurements. Later, M. H. Cohen (personal communication) reported similar anomalies in some Y-based oxides. In the absence of zero resistivity and evidence for the Meissner effect, the sharp resistance drops have been taken only as an indication of the possible existence of superconductivity at these unusually high temperatures. More recently, Chen *et al.*<sup>2</sup> detected the reverse a.c. Josephson effect in mixed-phase Y-Ba-Cu oxide samples below 240 K, providing the strongest evidence for superconductivity to date. Here we report the observation of a sharp decrease in resistance of at least four orders of magnitude at  $\sim 230 \text{ K}$  in one annealed sample of  $\text{EuBa}_2\text{Cu}_3\text{O}_{6+\delta}$ . Subsequent magnetic measurements on the same sample indicated a magnetic anomaly at the same temperature.

The  $\text{EuBa}_2\text{Cu}_3\text{O}_{6+\delta}$  sample was synthesized by a one-step solid-state reaction of appropriate amounts of  $\text{Eu}_2\text{O}_3$ ,  $\text{BaCO}_3$  and  $\text{CuO}$ . The powders were pressed into pellets which were then heated in a reduced oxygen atmosphere ( $2,000 \mu\text{m O}_2$  partial pressure) at  $925^\circ\text{C}$  for 4 h and cooled at room temperature. The X-ray diffraction patterns show that the sample displays only an orthorhombic phase typical for the 1-2-3 compounds and no phase transformation has been observed from 77 K. Based on our Mossbauer measurement, no anomaly has been observed from 300 K down to 4 K. This 90-K superconductor was pumped at 300 K and then exposed to argon for 48 h. The resistance of the sample was measured using a conventional four-point probe technique. The sample has a length of  $\sim 3 \text{ mm}$  and a cross-section of  $\sim 1 \times 2 \text{ mm}$ . The results at 1 mA are displayed in Fig. 1. The onset of the sharp drop in resistance (from  $\sim 1 \Omega$ ) occurs at  $\sim 238 \text{ K}$  and the resistivity reaches the zero-baseline ( $\leq 10^{-4} \Omega$ —our experimental resolution) at



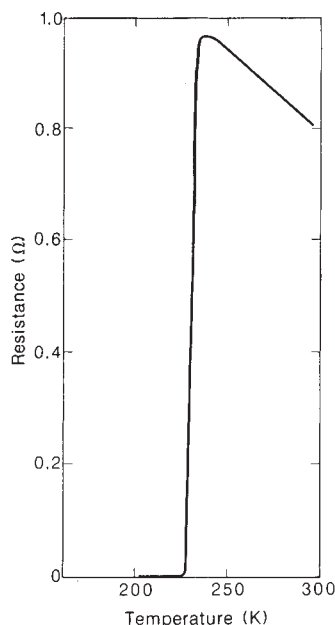


Fig. 1 Resistance plotted against temperature curve for mixed-phase  $\text{EuBa}_2\text{Cu}_3\text{O}_{6+\delta}$ .

~228 K. The midpoint of the drop is at ~230 K. A similar sharp drop in resistance at 0.1 mA was reproduced the following day. (The smaller current was used to avoid damaging the sample as we had done previously.) We interpret this sharp drop as a superconducting transition. However, the zero-resistance phenomenon at 230 K disappeared after several thermal cyclings, even though a small kink appeared at ~230 K. The kink disappeared completely after four weeks and the sample became an average 90-K superconductor. We point out that we have prepared several samples in the same manner as described but none has exhibited zero resistance at 230 K, even though some of them have somewhat higher superconducting transition temperatures.

To confirm the nature of this transition, it was necessary to measure the magnetization,  $M$ . The same sample was taken to the National Magnet Laboratory at Massachusetts Institute of Technology (MIT) one week later to use the SHE Model 905 SQUID magnetometer. Figure 2 shows the magnetization data below 90 K taken at 100 G. The temperature dependence shows that the superconducting transition is rather broad in comparison with a good, unpumped 90-K sample of  $\text{EuBa}_2\text{Cu}_3\text{O}_{6+\delta}$ <sup>3</sup>. The diamagnetic magnetization at 10 K is only ~33% of that of perfect diamagnetism.

The high-temperature data measured at 100 G are shown in Fig. 3. The magnetization at 300 K is proportional to the applied field up to  $5 \times 10^4$  G, demonstrating that the sample is paramagnetic. On cooling, the magnetization of the sample increases with decreasing temperature, expected for a paramagnet. But at 230 K, the rate of increase suddenly decreases, suggesting the onset of a diamagnetic component due to the Meissner effect in the magnetization. This onset temperature coincides with the occurrence of the resistance drop as shown in Fig. 1. The appearance of both 'zero resistance' and diamagnetism strongly suggests the onset of superconductivity at ~230 K. The observed magnetic anomaly is reversible. We have been able to reproduce this anomaly even at 900 G. Although the diamagnetic signal is clear, its small magnitude suggests that the 230-K superconductivity may arise from 'percolated' superconducting linear chains occupying only a small fraction of the sample (a few parts in  $10^4$ ). In the absence of any anomaly based on the Mossbauer measurement, which could detect both the structural and

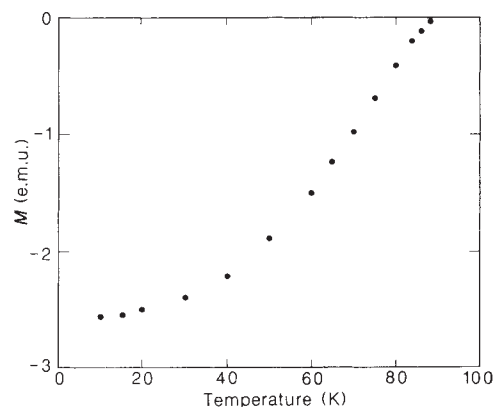


Fig. 2 Magnetization,  $M$ , at 100 G plotted against temperature,  $T < 90$  K.

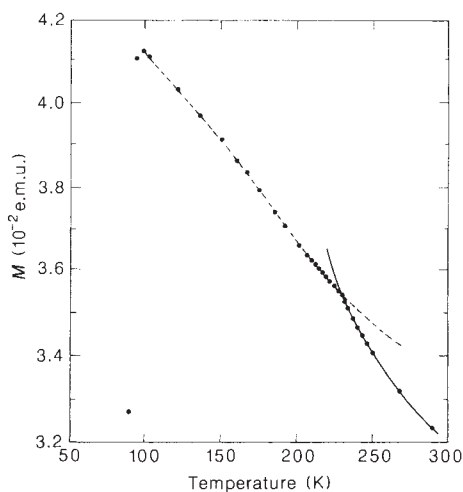


Fig. 3 Magnetization,  $M$ , at 100 G plotted against temperature,  $T > 90$  K. The solid and dashed curves are guides for the eye.

semiconductor-metal transformations and because of no example (except<sup>4</sup>  $\text{V}_{2-x}\text{Cr}_x\text{O}_3$  over a limited  $x$  range) exhibiting a semiconductor-metal transition on cooling, we rule out the interpretation of this anomaly in terms of a semiconductor-metal transition. The diamagnetic component has persisted through several thermal cyclings for more than three weeks.

The observations reported here, and in refs 1 and 2, appear to require the existence of a new structural or compositional phase which is different from the 1-2-3 90-K compound, independent of the trivalent element involved. We would also like to point out that the superconducting phase reported here may not be the same as those reported in refs 1 and 2. More experiments are necessary to obtain this high-temperature superconducting phase.

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# Kinetics of the BrO + ClO reaction and implications for stratospheric ozone

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The gas-phase reaction between BrO and ClO has been proposed as a potentially fast and synergistic mechanism of stratospheric ozone destruction. Further, it has been advanced<sup>1</sup> as a contributing factor to Antarctic springtime ozone column losses of ~40% from 1960 to 1985<sup>2,3</sup>. Both dynamical<sup>4</sup> and chemical theories<sup>1,5-8</sup> have been advanced to explain the formation of the Antarctic 'ozone hole'. A major uncertainty in these theories has been the rate constant and product distribution of the BrO + ClO reaction as a function of temperature. Here we report the first direct measurements of these parameters. We show that this reaction could, indeed, account for a large fraction of the springtime ozone depletion over Antarctica and provide a source of chlorine dioxide of sufficient magnitude to explain the recent measurements of this species in the Antarctic stratosphere, provided that the stratosphere contains a sufficient quantity of bromine (~20 p.p.t.v.).

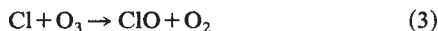
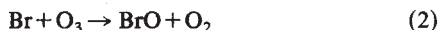
The reaction of BrO with ClO has the following accessible product channels:



The sets of products Br + ClOO and BrOO + Cl are also possible, but we do not distinguish these from channel (1b) because of the rapid thermal dissociation of ClOO and BrOO to halogen atoms and molecular oxygen, both in our laboratory experiments and in the stratosphere.

We performed the kinetics investigations using the technique of discharge-flow mass spectrometry<sup>9,10</sup> using the apparatus described in a recent publication<sup>11</sup>. Figure 1 is a schematic diagram of the apparatus configuration. Selected ion monitoring measured the concentrations of all molecular species (for example, Cl<sub>2</sub>, ClO, OClO, Cl<sub>2</sub>O, Br<sub>2</sub>, BrO, BrCl, NO and NO<sub>2</sub>) during each experiment.

BrO and ClO were produced in separate flow streams via microwave discharge of trace Br<sub>2</sub> and Cl<sub>2</sub> in He to produce Br and Cl atoms, respectively, which were then reacted with ozone:



To determine whether the means of producing ClO influenced the experimental results, ClO was produced in two additional ways, by reaction of chlorine atoms with OClO and with Cl<sub>2</sub>O:



Ozone concentrations were measured by reaction with excess NO



to substitute a stoichiometric amount of NO<sub>2</sub> for O<sub>3</sub>. Absolute

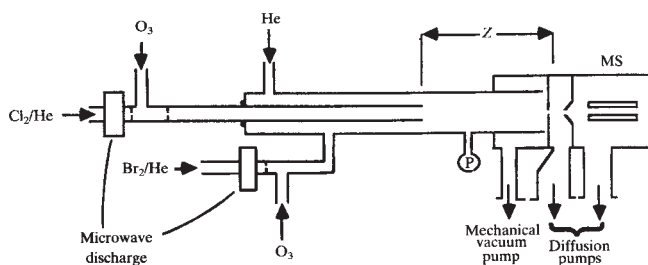


Fig. 1 Discharge-flow apparatus as configured for measurement of the rate constant and product distribution of the BrO + ClO reaction. ClO radicals are produced in the moveable inlet by microwave discharge of Cl<sub>2</sub> diluted in He and reaction of the Cl atoms with O<sub>3</sub>. BrO radicals are produced analogously by reaction of Br with O<sub>3</sub> and are introduced to the flow tube via a fixed inlet. Reaction time is varied by moving the ClO source relative to the first sampling orifice of a differentially pumped quadrupole mass spectrometer. Reaction time is computed from this distance, Z, and the velocity of the carrier gas. The latter is established by a mechanical vacuum pump and is calculated from measurements of the total flow rate of gases into the flow tube, the cross-sectional area of the flow tube, and the pressure and temperature of the reaction zone.

calibration of NO<sub>2</sub> as an ion current at  $m/e = 46$  was achieved by admitting known concentrations to the flow tube. The concentrations of ClO and BrO radicals were measured analogously to that of ozone via the reactions



and



Again, an excess of NO results in stoichiometric substitution of NO<sub>2</sub> for the species of interest, here either ClO or BrO.

The flow tube and both inner and outer surfaces of the movable injector were coated with phosphoric acid to reduce wall losses of reactive species. First-order losses of ClO and BrO on the flow-tube wall were measured at various times during this work and were always found to be negligible (<2 s<sup>-1</sup>). All of the kinetics measurements were made under pseudo-first-order conditions, [ClO]<sub>0</sub> >> [BrO]<sub>0</sub>. This condition was checked via measurement of the ClO and BrO concentrations and by observing the linearity of pseudo-first-order decay plots. Helium served as the carrier gas, and the total pressure in the flow tube was in the range 1.1–1.5 torr.

A typical decay plot showing the loss of BrO in the presence of excess ClO is shown in Fig. 2. The upper plot shows BrO measurements taken with the ClO discharge turned off. This blank is performed to account for BrO destruction due to any heterogeneous loss of BrO on the injector surface. At all temperatures the slope of this plot was <1 s<sup>-1</sup>, indicating negligible BrO surface loss. The lower plot shows the change in BrO signal as a function of increasing BrO + ClO reaction time. The negative value of the slope of a plot of ln(BrO) against time is a first-order rate constant,  $k^1$ . The bimolecular rate constant was calculated

Table 1 Data summary for the rate coefficient measurements

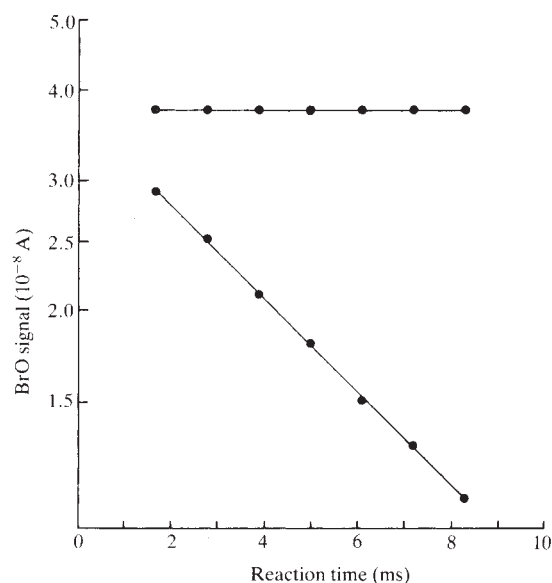
| Number of measurements | Temperature (K) | $k$ (10 <sup>-12</sup> cm <sup>3</sup> molecule <sup>-1</sup> s <sup>-1</sup> )* |
|------------------------|-----------------|----------------------------------------------------------------------------------|
| 32                     | 241             | 8.0 ± 1.1                                                                        |
| 69                     | 305             | 8.4 ± 1.0                                                                        |
| 13                     | 408             | 8.3 ± 0.8                                                                        |

Average value: 8.2 ± 1.0 (10<sup>-12</sup> cm<sup>3</sup> molecule<sup>-1</sup> s<sup>-1</sup>) where the uncertainty corresponds to the 95% confidence level and includes both random and possible systematic errors.

\* Error shown is one standard deviation (precision only).

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**Fig. 2** Typical kinetics data for the BrO + ClO reaction. The upper plot is the variation in BrO ion current as a function of movable inlet position with the ClO source turned off. The lower plot shows the decrease in BrO signal as a function of injector position in the presence of excess ClO. Reaction conditions:  $P = 1.30$  torr,  $T = 305$  K,  $[\text{ClO}] = 1.58 \times 10^{13}$  molecule  $\text{cm}^{-3}$ . The measured value of  $k^1 = 142.7 \text{ s}^{-1}$  gives the result  $k_1 = 9.0 \times 10^{-12} \text{ cm}^3 \text{ s}^{-1}$  for this particular run.

for each kinetics run using the equation

$$k = \frac{k^1}{[\text{ClO}]}$$

Table 1 summarizes the results of 114 measurements of the rate constant for reaction 1 made in the temperature range 241–408 K. Within experimental error, the rate constant,  $k_1$ , did not vary with temperature. Taking into account both random and possible systematic errors, we report  $k_1 = (8.2 \pm 1.0) \times 10^{-12} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$  for the total rate constant for the reaction forming all possible products.

The branching ratio into channel 1a, Br + OCIO products, was measured by first calibrating ClO relative to OCIO. The reaction



was used in these calibrations. Ion currents at  $m/e = 67$  and 51 were monitored as the  $\text{Cl}_2$  discharge (Cl source) was turned on and off. The  $\Delta m/e = 67$  signal was directly proportional to  $[\text{OCIO}]$  change resulting from reaction 4. The  $\Delta m/e = 51$  signal was the result of both changes in  $[\text{ClO}]$  and  $[\text{OCIO}]$  as the Cl source was modulated, because OCIO fragments by  $\sim 50\%$  into ClO. The  $m/e = 51$  signal was corrected for OCIO contribution using the method of Birks *et al.*<sup>9</sup>. The branching ratio into channel 1a was measured by monitoring  $\Delta[\text{OCIO}]/\Delta[\text{ClO}]$  as BrO was turned on and off. The BrO source was modulated either by turning the  $\text{Br}_2$  discharge on and off or by leaving it

on but interrupting the flow of  $\text{Br}_2$  through the discharge.

The branching fraction into channel 1c,  $\text{BrCl} + \text{O}_2$  products, was measured in a similar manner to that of channel 1a. The value  $\Delta[\text{BrCl}]/\Delta[\text{ClO}]$  was measured as BrO was modulated. To measure the relative sensitivities of BrCl at  $m/e = 116$  and ClO at  $m/e = 51$ , either excess  $\text{Br}_2$  or excess  $\text{O}_3$  was added to a stream of chlorine atoms to produce equal amounts of BrCl or ClO, respectively, according to the reactions:



The BrCl/ClO sensitivity ratio was determined over a wide range of concentration by varying the flow rate of  $\text{Cl}_2$  through the microwave discharge. Modulation of the BrO source in the presence of ClO was found to result in a large modulation of the BrCl ion current at  $m/e = 116$ . However, this was found to be due to a secondary reaction of Cl atoms produced in channel 1b with  $\text{Br}_2$  (reaction 9). The corresponding reaction of Br with  $\text{Cl}_2$  is endothermic by  $5.7 \text{ kcal mol}^{-1}$  and thus does not contribute to the BrCl signal. Addition of isobutane to the flow tube allowed Cl atoms to be removed from the reaction system. Under these conditions, no BrCl product could be detected.

The fast secondary reaction of Cl with  $\text{Br}_2$  (reaction 9) allowed channel 1b to be quantified. By using a sufficiently large concentration of  $\text{Br}_2$  ( $6 \times 10^{13}$  molecules  $\text{cm}^{-3}$ ), reaction 9 was forced to completion, and the branching ratio into channel 1b

**Table 2** Summary of product distribution experiments

| Temperature (K)                                            | Number of experiments | Reaction products              | Branching ratio ( $\pm \sigma$ ) |
|------------------------------------------------------------|-----------------------|--------------------------------|----------------------------------|
| 305                                                        | 18                    | (1a), Br + OCIO                | $0.53 \pm 0.04$                  |
| 241                                                        | 7                     | (1a), Br + OCIO                | $0.50 \pm 0.01$                  |
| 305                                                        | 9                     | (1b), Br + Cl + O <sub>2</sub> | $0.44 \pm 0.02$                  |
| 241                                                        | 10                    | (1b), Br + Cl + O <sub>2</sub> | $0.42 \pm 0.05$                  |
| 305                                                        | 4                     | (1c), BrCl + O <sub>2</sub>    | 0.00                             |
| Temperature averaged, sum of: (1a) + (1b) + (1c) = 0.95    |                       |                                |                                  |
| Temperature averaged branching ratios, normalized to 1.00: |                       |                                |                                  |
|                                                            |                       | Br + OCIO                      | $0.55 \pm 0.10^*$                |
|                                                            |                       | Br + Cl + O <sub>2</sub>       | $0.45 \pm 0.10^*$                |
|                                                            |                       | BrCl + O <sub>2</sub>          | $\leq 0.02^*$                    |

\* Uncertainty includes  $\sigma$  and an estimate of possible systematic errors.

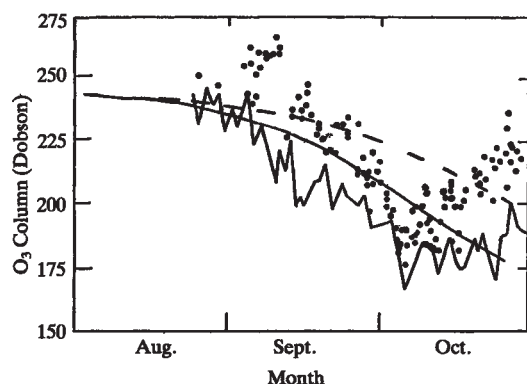


Fig. 3 Calculated time-dependent behaviour of the ozone column above 16 km at 80° S according to the model of Rodriguez *et al.*<sup>8</sup> A mixing ratio of 20 p.p.t.v. inorganic bromine is assumed. Solid line: results using the rate constant of Clyne and Watson<sup>12</sup> for channel 1a of the BrO+ClO reaction; dashed line: results using the rate constant reported here. Model calculations do not allow ozone depletion due to photolysis of the ClO dimer. Satellite data<sup>3</sup> for 74° S–78° S are observations over Halley Bay (points) and the minimum over all longitudes (jagged line).

is given by  $\Delta[\text{BrCl}]/\Delta[\text{ClO}]$  upon modulation of the BrO source.

The measured branching ratios are summarized in Table 2. Independent measurements of channels 1a and 1b added to 95%, invariant of temperature in the range 241–408 K. The 5% discrepancy is certainly within the experimental error of the measurements. We attribute all of the products to channels 1a and 1b and recommend a branching ratio of  $0.55 \pm 0.10$  for channel 1a and  $0.45 \pm 0.10$  for channel 1b. Complete details of the measurements of the rate constant and product distribution for this reaction, including an analysis of all possible interfering reactions, will be reported elsewhere.

Combining the results of the measurements of the rate constant for product loss with the branching ratio for channel 1b gives a value of  $3.7 \times 10^{-12} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$  for  $k_{1b}$ . This is 45% smaller than the value of  $6.7 \times 10^{-12} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$  measured by Clyne and Watson<sup>12</sup> and used in recent model calculations<sup>1,8</sup>. In the pioneering work of Clyne and Watson, ClO and BrO were produced simultaneously by the reaction

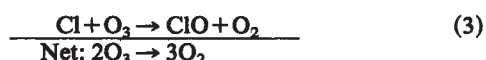
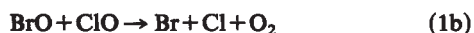


and the decays of BrO, ClO and OCIO monitored with time. Because reaction 10 is the reverse of reaction 1a, an equilibrium among the species of this reaction was approached. The lack of pseudo-first-order kinetics and interference from the reaction



also compromise the accuracy of this earlier study, which required computer modelling to extract the rate constant and branching ratios. Considering these complications in interpretation of the earlier work, the agreement of our work with that of Clyne and Watson is quite good.

Reaction 1 is of significance to the Antarctic stratosphere in that (1) it could provide a rapid means of ozone depletion, and (2) it could explain the recent observation of OCIO in this region of the atmosphere<sup>14</sup>. The catalytic cycle



is particularly significant in that it does not require sunlight or atomic oxygen to destroy ozone. A major difficulty of all theories of the Antarctic ozone hole based on chlorine-catalysed ozone

depletion is the low concentrations of ClO expected for this region of the atmosphere. Until recently, the predominant forms of chlorine in the polar stratosphere were predicted by atmospheric models to be chlorine nitrate and HCl. Some theories invoke heterogeneous reactions on stratospheric cloud particles<sup>1,5,8</sup> to convert oxides of nitrogen to nitric acid. Other theories rely on the condensation of nitric acid itself to form the polar stratospheric clouds<sup>6,7</sup>. In this way the ClO concentration of the polar stratosphere is increased. In any case, measurements made by de Zafra during the 1986 National Ozone Expedition showed anomalously high ClO in the lower stratosphere of Antarctica<sup>13</sup>.

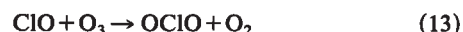
In Fig. 3, computer model results, incorporating our new value for  $k_{1b}$ , are compared with earlier model results (see ref. 8 for details of the model) using the rate constant of Clyne and Watson and with observations of the ozone column<sup>3</sup>. Although our reduced rate constant results in less predicted ozone depletion, the possibility of a large fraction of the observed ozone depletion being due to the BrO+ClO reaction is still indicated if there is as much as 20 p.p.t.v. total inorganic bromine there.

The recent and unprecedented observation of OCIO in the Antarctic stratosphere<sup>14</sup> and of its diurnal variation indicate that ClO is undergoing rapid cycling in this region of the atmosphere. The observed total column amounts of OCIO were found to be about 20–50 times larger than would be expected for standard homogeneous photochemistry. Furthermore, the BrO+ClO reaction is the only gas-phase reaction demonstrated to yield OCIO as a product. Thus, the observation of OCIO in the stratosphere is strong circumstantial evidence for channel 1a of the BrO+ClO reaction. If this channel is operative, the ozone destruction channel, channel 1b, is also operative.

We caution that other, as yet unknown reactions could possibly be the source of OCIO. In particular, the reactions



and



have been shown to be extremely slow in the gas phase<sup>9</sup>, but could possibly take place on the surfaces of polar stratospheric clouds. Assuming a particulate surface area<sup>7</sup> of  $10^{-7} \text{ cm}^2 \text{ per cm}^3$ , the time constant for collision of any molecule with an aerosol particle is about 1 h. It was reported earlier that reaction 13 does take place on the surface of an uncoated silica flow tube at 298 K<sup>9</sup>. However, the surface-to-volume ratio in that reactor was  $\sim 10^7$  times larger than that of polar stratospheric clouds. Molina and Molina<sup>15</sup> have postulated the photolysis of the ClO dimer both as a possible source of OCIO and as a catalytic reaction for ozone destruction. However, the photolysis products of  $(\text{ClO})_2$  have not yet been determined.

With respect to effects on ozone, the fate of OCIO produced in reactions 12 and 13 would most often be photolysis to regenerate ClO and produce atomic oxygen, which in turn reforms ozone. Therefore, these reactions, while producing OCIO, would be expected to have little effect on the ozone column. Laboratory studies of these and other heterogeneous reactions that might produce OCIO are called for. But such reactions remain speculative. Based on our present knowledge of reactions of chlorine species, the BrO+ClO reaction appears to be a potentially important reaction contributing to the formation of the ozone hole.

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## High concentrations of chlorine monoxide at low altitudes in the Antarctic spring stratosphere: diurnal variation

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The Stony Brook ground-based remote sensing mm-wave spectrometer was used to measure chlorine monoxide in the stratosphere over McMurdo Station, Antarctica during the austral spring of 1986. From the data collected, we find strong evidence for abnormally high concentrations of ClO at low altitudes—as much as two orders of magnitude greater than standard theories predict at 20-km altitude at mid-latitudes. This low-altitude ClO changes cyclically on a diurnal basis, and also secularly during the September–October observing period. A study of the diurnal variation of the low-altitude ClO is presented here. We conclude that chlorine is crucially involved in the springtime destruction of Antarctic ozone.

The recently discovered and rapidly increasing depletion of ozone in the Antarctic spring seasons since the late 1970s<sup>1,2</sup> has led to the suggestion of a variety of mechanisms to explain the phenomenon. Chemical theories involving chlorine have been likely candidates, due to the steady increase in chlorocarbons and chlorofluorocarbons reaching the stratosphere over the same time span. Other factors, so far unique to Antarctica, must also come into play however, or the observed large seasonal depletion of ozone would be seen on a worldwide basis.

The observation of large concentrations of ClO in the lower stratosphere (12–22 km) where the major depletions associated with the anomalous ozone hole are occurring<sup>3</sup> would be strong evidence that chlorine chemistry is implicated in the phenomenon (perhaps augmented, but not replaceable, by other proposed mechanisms (for example refs 4–6)). Here we present evidence that just such large concentrations of ClO, exhibiting a strong diurnal variation, do exist at low altitudes during the Antarctic spring.

The presence of chlorine monoxide (ClO) in the middle to upper stratosphere is a direct indication of the catalytic conversion of O<sub>3</sub> to O<sub>2</sub> by chlorine. ClO has a mixing ratio profile determined by the balance between a number of photolytic and chemical reactions<sup>7</sup>. Under normal stratospheric conditions, the mixing ratio peaks at ~38 km<sup>8</sup> and decreases rapidly with decreasing altitude below this level, at a rate of roughly 30% per km. The normal mixing ratio of ClO is predicted to be ~10<sup>-12</sup> at 20 km at tropical and temperate latitudes. One *in situ* balloon measurement exists down to ~22 km where ClO mixing ratios of ~10<sup>-11</sup> have been established<sup>8</sup>. In past ground-based mm-wave emission spectroscopy measurements<sup>9,10</sup> we have been able to determine the chlorine monoxide mixing ratio profile down to ~28 km, below which the combination of emission-line pressure broadening and low mixing ratio yield a line-shape contribution too broad and weak to be reliably detected.

The chlorine-related ozone depletion theories for Antarctica have generally invoked various heterogeneous (surface) reactions on aerosols or 'ice' grains in an effort to speed up the rate of ozone destruction, as conventional gas-phase reactions have been shown<sup>11–13</sup> to be too slow to account for the average rate of depletion observed in the Antarctic spring. New reaction routes involving ClO dimer formation<sup>14</sup> and BrO + ClO reactions<sup>11</sup> have also been proposed to augment the rate of ozone depletion. The purely chemical theories also require low concentrations of NO<sub>x</sub>, assumed to follow from sequestration of nitrogen in HNO<sub>3</sub> during the polar night. This prevents the suppression of active chlorine via reactions with NO<sub>x</sub>.

During the National Ozone Expedition of 1986 at McMurdo Station, Antarctica, frequent balloon soundings of ozone were made up to 30-km altitude by Hofmann *et al.*<sup>3</sup>. These showed that ozone depletion, at least in the outward reaches of the polar ozone hole typically observed over McMurdo during the spring of 1986, occurs in highly structured layers, and may reach >90% reduction in selected layers within two months after the Antarctic spring sunrise exposes the stratosphere to photolytic chemistry. These observations pose an even more severe requirement on the depletion speed than previously realized.

Here we concentrate on the relatively early time period from 1–22 September, to emphasize the observed ClO concentration

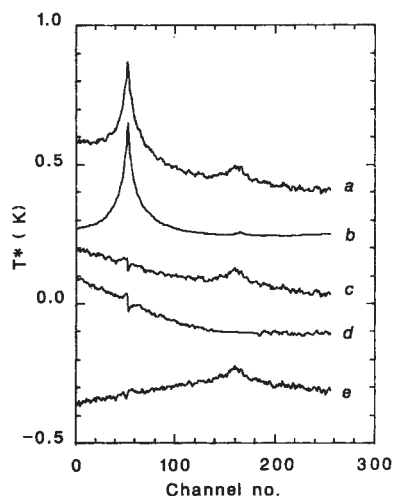


Fig. 1 Steps used in processing raw data, shown as curve (a). Curve (b) is a synthesized ozone background, including the  $v_2 = 1$ ,  $J = 18_{1,17} \rightarrow 18_{0,18}$  transition (left) and the much weaker  $v_1 = 1$ ,  $J = 14_{4,10} \rightarrow 15_{3,13}$  transition (near centre). Curve (c) = (a) - (b) and shows that residual instrumental curvature remains. Curve (d) is night-time data for >4 h after sunset at 45 km, with the remaining ClO signal suppressed. It contains the same instrumental curvature as (c). Curve (e) = (c) - (d).

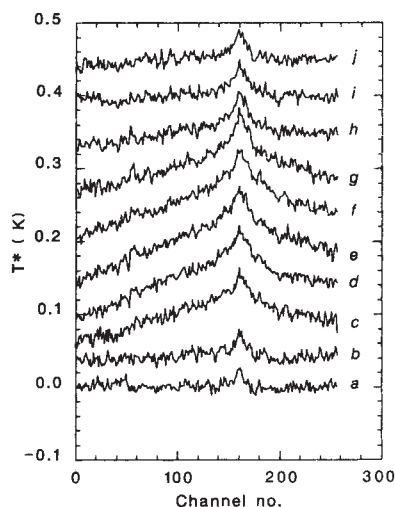


Fig. 2 Diurnal variation of ClO line-shape in 2-h time blocks. Curve (a) covers the last two night-time hours before dawn at 20 km, (b) the first two after sunrise, and so on. Curve (i) covers the first two hours after sunset at 45 km, curve (h) the preceding two hours, and so on. Curve (e), only, represents a variable length period bridging the midday interval, never more than four hours long.

and diurnal cycle during the period of most rapid ozone depletion. In a companion paper<sup>15</sup>, the secular change in ClO will be discussed over the interval from early September to late October. We have employed the Stony Brook ground-based mm-wave spectrometer at McMurdo Station (78° S) to observe the  $J=15/2 \rightarrow 13/2$  rotational emission line at 278.63 GHz (wavelength 1.1 mm). We have routinely measured stratospheric ClO at Mauna Kea, Hawaii and other locations for the past several years<sup>9,10,16</sup> using this same equipment.

The apparatus consists of a cryogenically cooled mm-wave heterodyne receiver feeding a 256 channel filterbank spectrometer which has a resolution of one MHz per channel. This bandwidth and resolution are sufficient to accurately measure the pressure-broadened emission line-shapes of molecules in the altitude range ~20–55 km.

Molecular rotational emission from low altitudes is strongly pressure-broadened: broadening decreases rapidly with increasing altitude. For molecular rotational spectra, Doppler broadening does not dominate over pressure broadening until altitudes above ~60 km. The observed pressure-broadened emission line-shape may be deconvolved to retrieve information about the concentration or mixing ratio of a given species as a function of altitude. We have used this technique to obtain diurnal changes in ClO mixing ratio as a function of altitude in the Antarctic spring.

Measurements of ClO at McMurdo began on 1 September 1986 and continued on a daily basis (except for brief periods of bad weather) until 29 October. At the beginning of this period, the stratosphere above 20 km was illuminated for about 10.5 hours per day; by 11 October for 24 hours per day. The time for photochemical reactions thus varied greatly. It is also important to realize that the Sun achieves a shallower slant angle of rise and fall with respect to the horizon as the period of continuous daylight approaches. Thus, considering the altitude span from 15 to 45 km, sunrise reaches the top of this column nearly an hour earlier than the bottom by early October, and the reverse time lag occurs at sunset. In time-sequencing our data to study the diurnal ClO cycle, we have arbitrarily defined sunrise as that time when sunlight first reaches 20 km, and sunset as the time when illumination ceases at 45 km.

Data were taken in successive 20-min observations and later averaged into two-hour time bins for the present study. Since the radiated intensity from the entire stratospheric ClO layer is very weak, ~0.01 to 0.1 K, from the zenith direction in equivalent Rayleigh-Jeans temperature units—the signal-to-noise ratio in a single 20-min observation is poor. Even in two-hour time bins, data must be averaged over a number of days to achieve an acceptable signal-to-noise ratio for satisfactory analysis of the diurnal variation. All of the analysis in this paper is based on

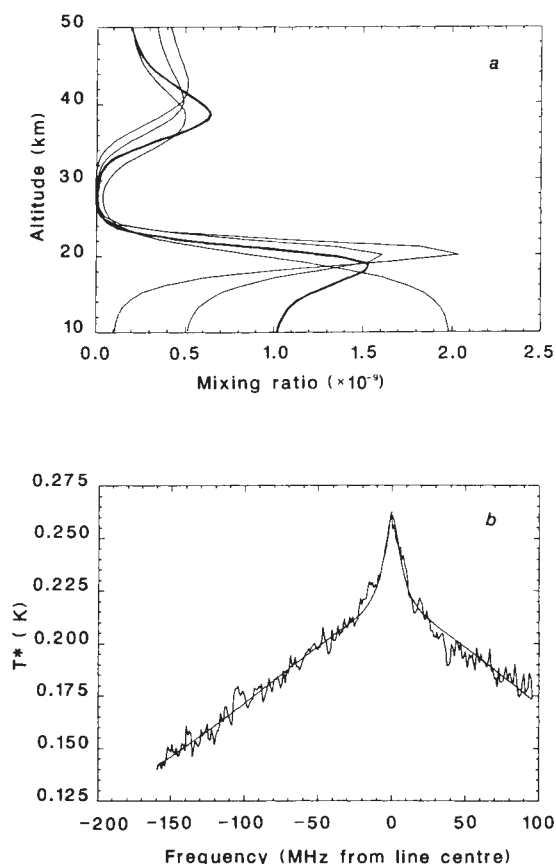
data averaged over the period 1–22 September 1986. There was some secular decline in low-altitude ClO during this period as discussed in a companion paper<sup>15</sup>: thus, conclusions presented here concerning mixing ratio versus altitude represent the average derived over the first three weeks of September.

The spectral window containing the ClO signal at 278.631 GHz also contains a well-documented<sup>17</sup> background of O<sub>3</sub>-line radiation, including one weak line (both peak and integrated intensities ~1/40 that of the stronger O<sub>3</sub> line in the passband) only 4 MHz away from the ClO line centre. There is negligible spectral interference from other species in this window<sup>17</sup>. The modelled O<sub>3</sub> line background is illustrated in Fig. 1b. It contains contributions from a total of 20 O<sub>3</sub> lines, most of whose centre frequencies lie well outside the spectral passband. The most prominent feature within the passband is the  $18_{1,17} \rightarrow 18_{0,18}$  rotational transition of vibrationally excited ( $v_2 = 1$ ) <sup>16</sup>O<sub>3</sub> centred at 278.521 GHz. The intensity of this line is strongly temperature-dependent, varying as  $T^{-3/4} \times e^{-1,200/T}$ , so that its pressure-broadened line-shape is dependent not only on concentration, but also temperature as a function of altitude. As the ClO emission line lies in one wing of this O<sub>3</sub> line, its underlying shape must be carefully determined and removed (along with other ozone line contributions) in order to recover the ClO line shape of importance in this study.

The following procedure was used to carry out this programme: a deconvolution of the core of the  $18_{1,17} \rightarrow 18_{0,18}$  ozone line was used to obtain an O<sub>3</sub> profile above ~25 km for each day. Daily temperature and pressure profiles for McMurdo, obtained from the National Climate Analysis Center, were used in the deconvolutions. Balloon ozonesonde data from McMurdo (D. Hofmann, personal communication) was used to cover the altitude range <25 km: data were interpolated between the nearest measurements to cover days when no ozonesondes were flown. The overall O<sub>3</sub> profile was then combined with daily temperature profile data and used to synthesize the line-shapes and intensities of each of the O<sub>3</sub> lines (of varying temperature sensitivity) which contribute to the total ozone background. The data of each day and night were processed in this manner before averages were compiled for the period 1–22 September. The success of this programme may be judged by the nearly total removal of the  $18_{1,17} \rightarrow 18_{0,18}$  line in parts c and d of Fig. 1. Other O<sub>3</sub> lines potentially affecting the ClO spectral retrieval will, in general, be removed at least as effectively, as the  $18_{1,17} \rightarrow 18_{0,18}$  line has a relatively extreme temperature dependence. The averaging of daily 'cleaned' data over 1–22 September will further suppress random residual errors in the ozone background accruing from small errors in the daily ozone or temperature profiles employed.

After subtraction of the synthetic ozone background, the data





**Fig. 3** *a*, Result of four deconvolutions of the sum of curves *d*, *e* and *f* in Fig. 2 (midday data) to retrieve mixing ratio profiles. Profiles shown result from initial mixing ratio choices of 0.1, 0.5, 1.0 and 2.0 p.p.b.v., constant from 10–60 km, as input to the deconvolution algorithm. Because pressure broadening is  $\gg$  spectral bandwidth below  $\sim 15$  km, an initial choice of mixing ratio remains unaltered by the retrieval process at lowest altitudes. *b*, Midday input data compared with synthesized line-shape generated from retrieved mixing ratio profile emphasized in (*a*). Line-shapes synthesized from other profiles in (*a*) are nearly identical and would not be clearly distinguishable.

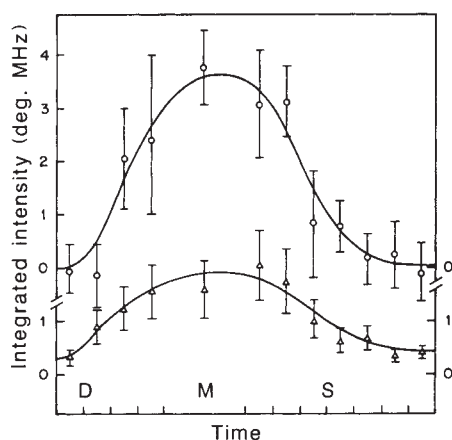
(Fig. 1c) still show a residual curved baseline which is partly of instrumental origin. To eliminate this instrumental curvature, a background was constructed by averaging night-time data (again with  $O_3$  removed as described above) starting from four hours after sunset until sunrise, (the time during which ClO is in low abundance through most of the stratosphere). The narrow (high-altitude) ClO line that remains in this night-time data was

suppressed by 'blanking' 20 MHz on each side of the line centre (Fig. 1d). The resulting night-time background was then subtracted from the data. We have used the same technique in a previous diurnal study of  $ClO^{10}$ .

Figure 2 illustrates the results for the ClO line-shapes within our spectrometer bandpass, after the data for the period 1–22 September was grouped into two-hour time bins and processed as described above. It is immediately apparent from Fig. 2 that starting after dawn (at 20 km), broad line wings are developed, signifying a rapid increase of low-altitude ClO. No such behaviour has been seen in our diurnal studies of ClO at  $20^\circ N$  latitude (ref. 9 and unpublished data). Less obvious from Fig. 2, the central portion of the line also grows in intensity after dawn, signifying that an increase in upper-level ClO also takes place. The overall line-shape in fact exhibits two components—the narrow line core and the much broader wings—which vary differently with time, suggesting that these arise from a bimodal vertical distribution of ClO with separate low- and high-altitude portions following somewhat different chemistry. A deconvolution study of the line-shapes of Fig. 2 bears out this analysis, as shown in Fig. 3. (The temperature dependence and transition strength from ref. 17 were used.)

Although the broad midday line wings require far greater than normal values for the mixing ratio at low altitudes to produce the observed line-shape, it is not possible to uniquely determine the required mixing ratio at or below  $\sim 20$  km from the data via deconvolution of the line-shape. This difficulty occurs because the pressure broadening is comparable to the full spectral bandpass in this altitude range, and the spectral line curvature contributed from that of a high mixing ratio of ClO at, for example 14–18 km, cannot be easily distinguished from that of a lesser mixing ratio at  $\sim 16$ –20 km, or from some intermediate combination. This feature may be seen in Fig. 3, where various choices for the initial mixing ratio used in the deconvolution algorithm are shown to be virtually insensitive to change in the 10–12 km range, while above this a small value of ClO at the lower altitudes is compensated for by more at  $\sim 20$  km, and a large value at the lowest altitudes results in substantially less at 20 km.

We are thus left certain of large diurnally varying low-altitude mixing ratio of ClO, but less certain of the detailed shape of the low-altitude profile or of the mixing ratio at a particular (low) altitude. Our best estimate, based on a number of trial deconvolutions, is that the average (1–22 September) mixing ratio at 20 km must be more than  $0.5 \times 10^{-9}$  and less than  $2 \times 10^{-9}$  at 20 km, assuming that the ClO extends from  $>20$  km down to at least 15 km and is not concentrated in a thinner layer. We also believe that  $\sim 1.5$  to  $2 \times 10^{-9}$  is an upper limit on the average mixing ratio  $<20$  km during the period covered here, based on the accuracy of the line-shape fits obtained. In any case, these values are two orders of magnitude more than standard chemical predictions at  $\sim 20$  km, giving a clear indication that unusual



**Fig. 4** Integrated line intensities from data as binned in Fig. 2. The abscissa is marked in 2-h intervals, except for the variable interval near midday. Points D, M and S represent dawn at 20 km, noon, and sunset at 45 km, respectively. Upper curve represents diurnal behaviour of low-altitude component only, integrated over  $\pm 100$  MHz around the line centre. Lower curve represents diurnal behaviour of high-altitude component only, integrated over  $\pm 50$  MHz around line centre. Note that limits imposed on low-altitude component omit a significant fraction of the actual line intensity. Curves are freehand fits to data. Error bars represent  $1\sigma$  (standard deviation) uncertainties of integrations using various fitting procedures to separate high- and low-altitude line components.

chlorine chemistry is occurring in the lower Antarctic spring stratosphere. (Additional quantitative discussion following from an alternative approach to defining the low-level component limits will be found in a companion paper<sup>15</sup>.)

Although it is difficult to recover quantitative data on the low-altitude mixing ratio beyond that stated above, other significant information is contained in the diurnal behaviour of the integrated line intensities for the low- and high-altitude components, respectively. We display the integrated intensities in the central narrow component and in the broad component separately in Fig. 4, as measured in two-hour time bins over the diurnal cycle. It should be noted that the frequency limits ( $\pm 100$  MHz) within which the low-altitude component has been integrated, omit a significant fraction of its total intensity. The high-altitude component decreases slowly with time after sunset and never disappears, showing a night/day ratio of  $\sim 0.2$ . This component also appears to decrease more slowly than seen in the diurnal cycle at  $20^\circ$  N latitude<sup>10</sup> and will be separately analysed in another paper. The low-altitude component decreases rapidly towards sunset. It undergoes negligible increase in the first 2-h period after dawn at 20 km (curve *b* of Fig. 2) and then appears very strongly within the second 2-h period. These features may be helpful in elucidating the chemistry involved, as discussed in a preliminary study Rodriguez *et al.*<sup>18</sup>.

The presence of another chlorine-bearing molecule, OCIO, has also been detected in the Antarctic spring stratosphere during the National Ozone Expedition<sup>19</sup>. Although again signalling the presence of anomalous low-altitude chlorine chemistry, current uncertainties in the formation and photolysis rates of this molecule, as well as the photolysis products, make it more difficult to use in quantitatively deducing the chemical pathways involved in lower stratospheric chlorine chemistry in Antarctica. The delayed post-dawn rise in low-altitude ClO, if sustained by further measurements, would limit the role of night-time Cl reservoir species such as OCIO which can be rapidly photolysed at long wavelengths.

The ClO data presented here, illustrating the major role being played by chlorine in the Antarctic spring ozone depletion at low altitudes, and the concurrent discovery of abnormally high concentrations of OCIO during the same time period over McMurdo, leave little doubt that the development of the Antarctic ozone hole involves chlorine chemistry as a direct and prominent feature.

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## High concentrations of chlorine monoxide at low altitudes in the Antarctic spring stratosphere: secular variation

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We have reported the observation of millimetre-wave emission from abnormally high concentrations of chlorine monoxide, ClO, in the lower stratosphere over McMurdo Station, Antarctica during the period 1–22 September 1986 and demonstrated<sup>1</sup> its diurnal behaviour. Here we report on the secular variation of daytime ClO in the lower stratosphere with a time resolution of 4 or 5 days, during the period 1 September to 16 October. We show that the ClO concentration declines during the latter half of September and disappears by early October. As ClO is directly involved in a catalytic ozone destruction cycle, these observations give evidence that the Antarctic ozone hole<sup>2</sup> is caused by a chemical depletion mechanism which operates in the early spring. This is also consistent with models in which large quantities of ClO are maintained through chemical reactions<sup>3–5</sup> on the surface of polar stratospheric cloud particles which evaporate with the warming of the stratosphere.

Our observations at a wavelength of 1.1 mm are carried out using a cooled mm-wave receiver and a 256-channel spectrometer. To obtain reliable spectra over the full bandwidth of the spectrometer (256 MHz) and remove instrumental effects, we take advantage of the diurnal cycle of ClO<sup>1,6</sup> and use day minus night spectra to discuss the secular behaviour. At McMurdo Station (latitude  $78^\circ$  S) there is no stratospheric sunset after about 6 October and less than two hours of night-time during the first few days of October. Thus it is not possible to subtract October night-time data from October daytime data. We have therefore used a single September night-time spectrum, averaging all data from 1–30 September (from four hours after sunset of two hours before sunrise) as the night-time background for all data. As discussed in ref. 1 each daytime and each night-time spectrum was processed separately before the averaging was carried out. In particular, the background contributed by ozone signals was subtracted out on a daily basis, separately for daytime and night-time spectra, leaving only the ClO signals. We have tested this averaging procedure to see if the day minus night spectra look substantially different in early September when only the nights immediately following the days in the bin are used; the shape of the day minus night spectrum remains very similar to that obtained using the September night-time average. The secular behaviour discussed here represents the changes in the ClO daytime spectra only. We define daytime as the period from three hours after sunrise to two hours before sunset. The daytime data have been binned approximately every four or five days (the minimum needed for an adequate signal to noise ratio), into 8 secular bins from 1 September to 16 October. The resulting binned data are thus each an average of four or five single daytime spectra minus the September night-time average, with the earliest period being 1–5 September and the last 13–16 October.

Figure 1 shows a progression of ClO spectral lines from 1 September to 12 October. Inspection of the line profiles shows the clear signature of low-altitude ClO during September in the form of strong line wings which appear at approximately constant strength in data from bins on 1–5 September, 10–13 September and 14–17 September. The broad line wings extend over the full 256-MHz bandpass of the observations, from 162 MHz below the line centre to 92 MHz above it. There is a gradual

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decline in the next two time bins, 18–21 and 23–25 September, that becomes obvious by 27 September–1 October. During the period 27 September–1 October and 8–12 October the line wings are virtually gone and the spectrum appears flat at frequencies  $>50$  MHz from the line centre. There is thus a strong secular trend in the strength of the ClO line wings. As the shape of the emission is determined by pressure broadening, this is evidence of substantial ClO (at high pressures) in the lower stratosphere in early and mid September, which is gone by early October. There appears to be little change in the line centre  $\pm 25$  MHz which arises from the middle and upper stratosphere. Figure 2 is a detailed comparison of the 14–17 September data with that from 8–12 October, the strongest and weakest emission lines observed. The integrated intensity under the line is  $\sim 5$  times greater during 14–17 September than during 8–12 October.

We have investigated the altitude distribution of ClO which is responsible for the early September emission, by deconvolving the pressure broadened spectral line profile using two different mathematical techniques<sup>7,8</sup>. An example of an altitude profile retrieval and a discussion of the limitations at low altitude are given in ref. 1. The results of many numerical experiments with a wide array of starting profiles for the mixing ratios show that (1) the retrieved altitude profile is always bimodal with one maximum below  $\sim 25$  km and another at  $\sim 40$  km, and (2) it is not possible to determine the precise shape of the lower stratospheric ClO profile except that it arises from substantial ClO below 25 km and has a mixing ratio in the range of 0.5 to 1.5 p.p.b.v. If, however, the true ClO distribution in the lower stratosphere extends only up to a maximum height of 20 km then the ClO mixing ratio can be as high as 2.5 p.p.b.v. ( $2.5 \times 10^{-9}$ ), and still give a good fit to the observed spectrum. This is clearly an extreme case as it requires that almost all of the available chlorine is in the form of ClO.

To demonstrate quantitatively the secular trend in the data, we have adopted a simplified model stratosphere by assuming that the spectral line wings arise from ClO which has a constant mixing ratio above 15 km and below an upper altitude  $h'$  that varies from 20 to 24 km. The altitude profile above 30 km is assumed to be the same over the entire period and has been obtained from the deconvolution of the central parts of the line; it has no significant effect on the derived mixing ratio in the lower stratosphere. The 'retrieval' process is then reduced to a best fit involving only one parameter for the lower stratosphere, the constant mixing ratio over the chosen altitude range. We obtained a best fit value for the mixing ratio at an altitude,  $15 < h < h'$  km for each of the eight periods between 1 September and 16 October using several values for  $h'$ . Figure 3a shows an example of the match between the data and the synthetic spectral line from the best-fit altitude profile shown in Fig. 3b, for the period 14–17 September.

Figure 4 shows the secular trend of the ClO mixing ratio using the above model. For an upper altitude  $h' = 22$  km, the ClO mixing ratio is  $\sim 1.4 \pm 0.3$  p.p.b.v. from 1–17 September and then gradually declines to  $\sim 0.5 \pm 0.3$  p.p.b.v. at the end of September and is near  $0.0 \pm 0.3$  p.p.b.v. during 8–12 October. If  $h'$  is 20 km instead of 22 km the mixing ratios are found to be  $\sim 1.7$  times larger, if  $h'$  is assumed to be 24 km they are  $\sim 1.7$  times smaller. We note that Hofmann *et al.*<sup>9</sup> have found most of the ozone depletion below 20 km over McMurdo during the same time span as these observations, although we have found evidence of some depletion up to  $h = 25$  km via mm-wave observations<sup>10</sup> at McMurdo.

The ClO mixing ratio in any of the models in Fig. 4 is two orders of magnitude greater during September than expected from normal chlorine chemistry as found at mid-latitudes. Although we cannot determine the precise distribution of the low-altitude ClO, the percentage decline of the ClO from early September to mid-October is independent of the assumed distribution, provided the actual shape of the altitude profile is not strongly time-dependent. With this assumption, the total lower

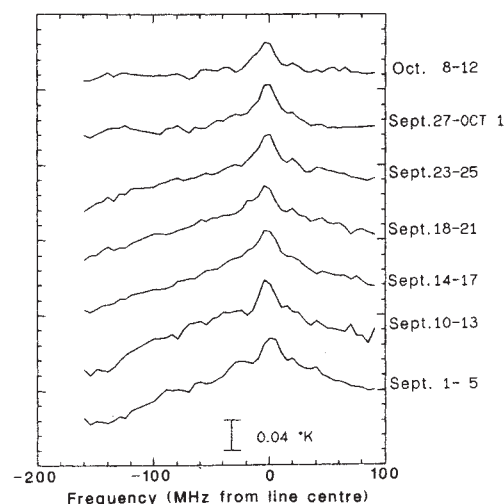


Fig. 1 Emission spectra scaled to the zenith direction for chlorine monoxide (ClO) obtained during September and October 1986. The central frequency is 278,630 MHz. The data, originally obtained with 1 MHz per channel, have been smoothed to a resolution of 5 MHz. The presence of ClO in the lower stratosphere is indicated by the emission in the line wings which extend out to  $\sim 162$  and  $+94$  MHz from the line centre. Towards the end of September and in October the shape of the emission has changed to a flat spectrum in the line wings indicating a disappearance of the lower stratospheric ClO. The intensity scale is equivalent Rayleigh-Jeans emission temperature.

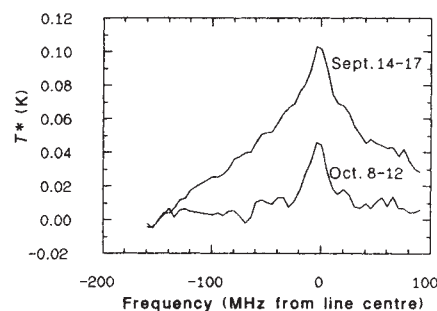


Fig. 2 A magnified picture of two ClO emission spectra from Fig. 1. The 14–17 September is a period of high concentration of ClO below an altitude of 24 km. The 8–12 October is a period with no observable low-altitude ClO. Both spectra have been set arbitrarily to an intensity of  $T^* = 0.0$  K at a frequency 150 MHz below the line centre.

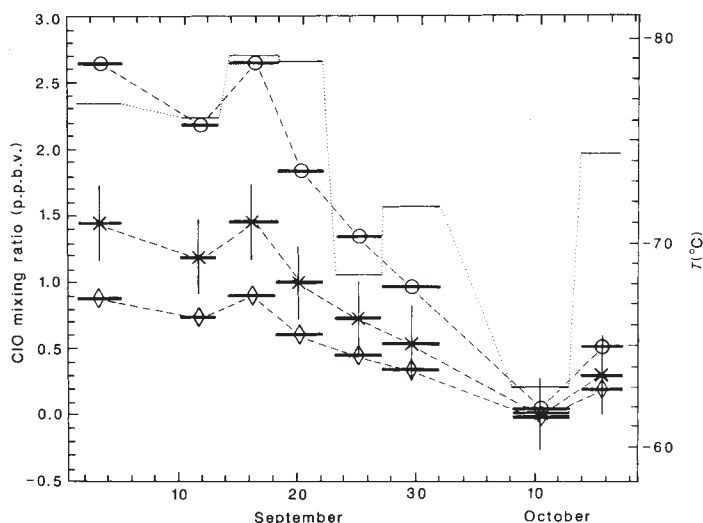
stratospheric ClO declined by about a factor of 5 or more from the first week in September until the second week in October.

A possible alternative interpretation of Fig. 4 is that the ClO layer is simply being reduced during September from the top down. Instead of a constant altitude distribution with decreasing average mixing ratio, the progression through September could result primarily from a lowering of the altitude at which ClO is present. In practice, both effects probably contribute to the decline in the observed signal strength.

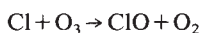
Observations of polar stratospheric clouds show a maximum altitude<sup>11</sup> of  $\sim 20$  km at the beginning of September, drifting down to  $\sim 17$  km by mid-September. These represent averages over Antarctica but if excess ClO exists only in the presence of polar stratospheric clouds that is, at or below 20 km, then the ClO mixing ratio required to match our data would be  $\geq 2.5$  p.p.b.v. which is close to the maximum chlorine content available. The drift downward of the clouds is in agreement with the alternative explanation of Fig. 4.

All of the chemical models<sup>3–5</sup> for springtime Antarctic ozone depletion involve heterogeneous chemical reactions on the sur-

**Fig. 4** The observed trend in lower stratospheric ClO over McMurdo Station, Antarctica during September and October 1986. The mixing ratios are assumed to be constant over the altitude range from 15 to 20 km (circles), 15 to 22 km (crosses) or 15 to 24 km (diamonds). The mixing ratios are averages over the dates covered by the bar. The uncertainties, given the assumed layer and constant mixing ratio, are  $\sim 20\%$  of the largest mixing ratio for each series. Thus for  $15 \text{ km} < h < 22 \text{ km}$ , the uncertainty is  $\sim \pm 0.3 \text{ p.p.b.v.}$  The data from 14–16 October are of poorer quality but are included for completeness. The dashed lines connecting the data are only meant as a guide. The thin solid lines connected by dots are the stratospheric temperatures at an altitude of 18 km.



face of particles in polar stratospheric clouds. These reactions release chlorine from the reservoir species  $\text{HCl}$  and  $\text{ClONO}_2$ , and lock up the odd nitrogen, resulting in a large excess of ClO which then takes part in one or more ozone depletion cycles all of which involve



We have shown that substantial lower stratospheric ClO is indeed present over McMurdo, Antarctica during September but disappears in October. The substantial low-altitude ClO is itself important evidence for ozone depletion at least in part by a chemical mechanism. The actual rate of ozone destruction in any chemical model depends on the rate at which the odd oxygen in ClO is converted to  $\text{O}_2$ , freeing the atomic chlorine to attack ozone. Two suggested mechanisms for this involve the ClO dimer<sup>5</sup> or the reaction<sup>4</sup> of ClO with  $\text{BrO}$ . Whatever the details of the reactions, the presence of large amounts of ClO at the same time and altitude as the ozone depletion suggests that the

destructive chlorine-chemistry cycle is efficient.

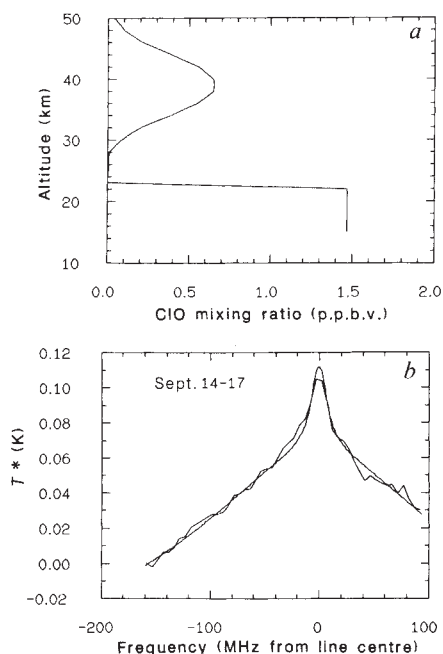
Another indication of unusual chlorine chemistry in the lower stratosphere over McMurdo Station, is the observation<sup>12</sup> of unusually large columns of night-time OClO during the same time span. The secular variation observed in OClO is consistent with that found here for ClO. In particular both species are observed to disappear on about 1 October, and the presence of both demonstrates that chlorine chemistry is highly anomalous during September in Antarctica.

We also note that the highest concentrations of ClO occur at the same time as the lowest stratospheric temperatures, which are required for the existence of polar stratospheric clouds<sup>13</sup>. The temperature (US Climate Analysis Center, personal communication) at the 18-km level (approximately 50-mb pressure) was on average  $-79^\circ\text{C}$  between 1–5 September,  $-77^\circ\text{C}$  between 10–13 September and  $-80^\circ\text{C}$  between 14–17 September, all periods of high ClO mixing ratios (see Fig. 4). Beginning 23 September the temperatures warmed to  $-70^\circ\text{C}$  and reached  $-61^\circ\text{C}$  during the second week of October. During the period 8–12 October when we see no evidence of ClO in the lower stratosphere the temperature at 18 km altitude was  $-63^\circ\text{C}$ .

In summary, the presence of ClO in early September and its disappearance in October reported here point towards chlorine as playing an important role in the origin of the springtime Antarctic ozone hole<sup>2</sup>. Although the specific models are speculative, there is sufficient ClO present at the right time and place to account for the Antarctic ozone depletion<sup>14</sup>, assuming current estimates for the rates of the processes, particularly the rate of photolysis of  $\text{Cl}_2\text{O}_2$  leading to atomic chlorine in the proposed catalytic cycles.

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**Fig. 3** *a*, The ClO mixing ratio altitude profile that was obtained from a best fit to the data of 14–17 September, assuming that ClO in a layer between 15 and 22 km was responsible for the strong emission in the spectral line wings (see text). *b*, The observed spectrum and synthetic spectrum resulting from the altitude profile in Fig. 3*a*.

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# Fate of US and Canadian combustion nitrogen emissions

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While approximately  $7.5 \times 10^{12}$  grams (7.5 Tg) of combustion nitrogen are emitted yearly in the United States and Canada<sup>1-3</sup>, only 1.5–2.3 Tg are deposited as acid rain over that same region<sup>4-6</sup>. Past nitrogen budget estimates<sup>4,10</sup> as well as recent observations<sup>11</sup> suggest that dry deposition and export play major roles. Here we report how the yearly accumulated deposition and transport of combustion nitrogen is simulated for the first time by a global transport model with realistic meteorology. The model predicts that dry deposition over the United States and Canada accounts for at least 2.1 and most probably 3.5 Tg of the emissions. The remainder is exported, principally over the North Atlantic. But at most 0.2 Tg, less than 3% of the estimated European emissions<sup>4</sup>, is predicted to reach Europe from North America. Furthermore, the model predicts that, while less than 40% of the nitrogen deposited as acid rain in the northeastern United States and eastern Canada comes from emissions in that region, almost all of the dry-deposited nitrogen and over half of the total acid nitrogen deposition comes from there.

Rather than simulations of individual storms and their resulting deposition and transport<sup>9</sup>, we are interested in the accumulated impact of nitrogen emissions. The Geophysical Fluid Dynamics Laboratory (GFDL) general circulation/transport model has 11 terrain-following levels with standard heights of 31.4, 22.3, 18.8, 15.5, 12.0, 8.7, 5.5, 3.1, 1.5, 0.5, 0.08 km, a horizontal grid size of ~265 km, and a time step of ~26 min. The 6-h time average winds and precipitation provided by the parent general circulation model compare well with observed global climatology<sup>7,8</sup>. This model also generates an ensemble of realistic weather patterns over North America that mimic real synoptic systems in their structure and evolution (W.J.M. & H.L.II, in preparation).

The gaseous and particulate nitrogen compounds resulting from both direct emissions and chemical transformations make up the collection of reactive nitrogen compounds that we call  $\text{NO}_y$ . These compounds are transported as a single tracer and satisfy the following continuity equation:

$$\frac{\partial R p_*}{\partial t} = \text{TRANSPORT} + \text{SOURCE} - D p_* R - \Phi p_* R \quad (1)$$

In equation (1)  $R$  is the  $\text{NO}_y$  volume mixing ratio,  $p_*$  is the surface pressure,  $D$  is the dry deposition coefficient, and  $\Phi$  is the precipitation removal coefficient. The formulation of TRANSPORT, which represents flux convergence resulting from both the resolved air motions and sub-grid scale diffusion, is described in detail elsewhere<sup>12-14</sup>.

SOURCE is constructed from yearly average point and area emission inventories<sup>1,2</sup> and held constant throughout the year. It includes an apportionment of point sources by stack height (ref. 3; A. Kosteltz, personal communication). Integrated over the source region, 65% of the emissions enter at the model surface, 20% at the middle of the bottom level (80 m) and 15% are injected into the second level at a standard height of 500 m. The distribution of total yearly emissions is shown in Fig. 1. The cross-hatched area, running northwards and eastwards from central Pennsylvania, delineates the source for the northeastern United States and eastern Canada and contains 15% of the total nitrogen emissions.

Chemical reactions<sup>15</sup>, by oxidizing insoluble nitrogen oxides to soluble nitric acid and by apportioning  $\text{NO}_y$  among the

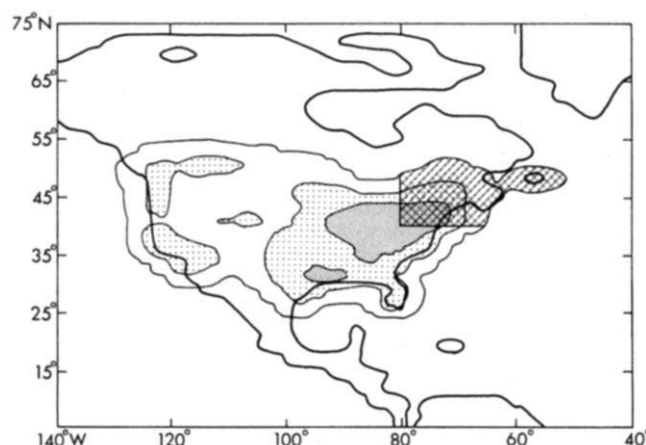


Fig. 1 Yearly total combustion nitrogen source ( $\text{mmol m}^{-2} \text{yr}^{-1}$ ) for the United States and Canada. □ 0–20  $\text{mmol m}^{-2}$ ; ▤ 20–100  $\text{mmol m}^{-2}$ ; ▨ 100  $\text{mmol m}^{-2}$ ; ■ North-east region.

Table 1 Combustion nitrogen budget for the United States and Canada

| Simulation             |     | Acid precipitation | Dry deposition (%) | Export (%)    |
|------------------------|-----|--------------------|--------------------|---------------|
| $w_{\text{eff}} +$     | $N$ | (% of emissions)   | of emissions)      | of emissions) |
| 0.3 cm s <sup>-1</sup> | 9   | 28%                | 46%                | 26%           |
| 0.1 cm s <sup>-1</sup> | 5   | 29%                | 28%                | 43%           |

nitrogen compounds with their differing deposition velocities, control the rate of acid deposition. However, chemical reactions only enter our model implicitly via the bulk removal coefficients,  $D$  and  $\Phi$ , discussed next. This is equivalent to assuming that the chemical partitioning is rapid relative to transport. While valid near the surface where the chemistry and removal are generally fast, this assumption starts to break down in the middle troposphere where the reactions can be much slower.

Dry deposition, a continuous process occurring only at the surface, is expressed as a first-order loss term in equation (1). The coefficient  $D$  is given in equation (2) of ref. 14 with  $w_0$  replaced by an effective deposition velocity,

$$w_{\text{eff}} = \frac{1}{R_{11}(\text{NO}_y)} \sum_i R_{11}(i) w(i) \quad (2)$$

that depends on the chemical partitioning of  $\text{NO}_y$ .  $R_{11}(i)$  is the volume mixing ratio of the  $i$ th reactive nitrogen species in the lowest level and  $w(i)$  is its measured deposition velocity. For a more detailed discussion, see refs 17 and 18. Using available measurements of  $w(i)$  and  $R(i)$ <sup>4,16-20</sup>, we find that, while values of both vary greatly with time and location,  $w_{\text{eff}}$  over land ranges from 0.6  $\text{cm s}^{-1}$  in the summer to 0.2  $\text{cm s}^{-1}$  in the winter.

With no chemical scheme in the model to calculate  $w_{\text{eff}}$ , we treat it as a parameter. Because we are primarily interested in yearly dry deposition, constant values, 0.3  $\text{cm s}^{-1}$  over land and 0.1  $\text{cm s}^{-1}$  over the ocean, are selected from the low end of the seasonal range. To determine the sensitivity of our conclusions to the variability and uncertainty in  $w_{\text{eff}}$ , simulations are also performed for  $w_{\text{eff}} = 1.0 \text{ cm s}^{-1}$ , an upper limit which assumes that  $\text{HNO}_3$  is the principal nitrogen compound, and for  $w_{\text{eff}} = 0.1 \text{ cm s}^{-1}$ , a lower limit which assumes that  $\text{HNO}_3 = 0.1 \text{ NO}_y$  and the ground is snow-covered.

Precipitation removal, an intermittent process acting throughout the tropospheric column, is also expressed as a first-order loss term in equation (1). The removal coefficient at level  $k$ ,  $\Phi_k$ , is given by

$$\Phi_k = N B_k [\text{Rain}/\text{Rain}^G] \quad (3)$$

where Rain is the 6-h model precipitation at a given surface

**Table 2** Comparison of observations and simulations for selected stations

| Station            | Precipitation $\text{cm yr}^{-1}$ |          | Acid nitrogen precipitation $\text{mmol m}^{-2} \text{yr}^{-1}$ |          |                        |
|--------------------|-----------------------------------|----------|-----------------------------------------------------------------|----------|------------------------|
|                    | Model                             | Observed | $w_{\text{eff}} = 0.3$                                          | Observed | $w_{\text{eff}} = 0.1$ |
| Oak Ridge TN       | 76                                | 128      | 18                                                              | 21       | 18                     |
| Oxford OH          | 62                                | 98       | 23                                                              | 24       | 23                     |
| Urbana IL          | 51                                | 88       | 25                                                              | 22       | 24                     |
| Charlottesville VA | 89                                | 106      | 33                                                              | 25       | 34                     |
| State College PA   | 107                               | 109      | 34                                                              | 33       | 37                     |
| Ithaca NY          | 113                               | 104      | 40                                                              | 30       | 44                     |
| Chalk River Ont.   | 93                                | 80       | 27                                                              | 24       | 29                     |
| Whiteface NY       | 129                               | 111      | 41                                                              | 21       | 44                     |
| Lewes DE           | 107                               | 118      | 26                                                              | 22       | 27                     |
| Brookhaven NY      | 117                               | 116      | 46                                                              | 22       | 46                     |
| Bermuda            | 190                               | 172      | 5                                                               | 6        | 6                      |
| Nova Scotia        | 125                               | 140      | 18                                                              | 14       | 20                     |
| Bay D'Espoir Nfld  | 103                               | 165      | 10                                                              | 9        | 12                     |
| Montmorency Que    | 128                               | 116      | 27                                                              | 25       | 30                     |
| Goose Bay Nfld     | 140                               | 94       | 11                                                              | 7        | 15                     |

grid box,  $\text{Rain}^G$  is the global average for the same period,  $1/B_k$  is a lifetime based on the vertical distribution of rainfall<sup>12</sup> and ranges from 20 days at the lowest level to 60 days at the 8.7 km level. The parameter,  $N$  represents the effective solubility of the trace species. The deposition of radioactive debris from nuclear tests in the stratosphere<sup>12</sup> has been successfully simulated with  $N = 1$ . A formulation for highly soluble gases, based on Henry's Law of solubility and a parameterization of cloud physics that includes both rainout in the cloud and washout below the cloud, reduces to a form similar to equation (3) (W.L.C., H.L.II and W.J.M., in preparation). Here,  $N$  depends on the fraction of  $\text{NO}_y$  present as gaseous  $\text{HNO}_3$  at the onset of precipitation and on the rate of conversion of insoluble nitrogen oxides to highly soluble nitric acid during the precipitation event. While the model has no chemical scheme to calculate the fraction and conversion rate, the percentage of emissions deposited yearly in acid rain over the United States and Canada is known (25%–30%<sup>4–6</sup>). The parameter  $N$  is set to bring the simulated yearly acid rain into this range. Based on recent observations<sup>24</sup>, we include no seasonal dependence. Since the level of  $\text{NO}_y$  increases with decreasing  $w_{\text{eff}}$  and the deposition of acid rain is fixed, the chosen value for  $N$  will decrease with  $w_{\text{eff}}$ . While the area integral depends on  $N$ , the spatial pattern of deposition as well as individual station values depend on the transport model's meteorology.

Fifteen-month simulations with the US-Canadian source, all using the same meteorology, were completed for three values of  $w_{\text{eff}}$  over land (1.0, 0.3, and  $0.1 \text{ cm s}^{-1}$ ) with  $N$  set at 18, 9, and 5 respectively. The apportionment of the combustion nitrogen emission among acid precipitation, dry deposition and export is given in Table 1 for the last two simulations. At the lower limit for  $w_{\text{eff}}$  ( $0.1 \text{ cm s}^{-1}$ ), dry deposition still equals deposition in acid rain. While export becomes the major term in the budget, only 3% of the emissions reach Europe. For the more probable value of  $w_{\text{eff}}$  ( $0.3 \text{ cm s}^{-1}$ ), dry deposition is the dominant term in the budget. The major portion of exported nitrogen is still carried out over the North Atlantic, though now only 1% of the emissions reaches Europe. Less than 0.1% survives the tropical rainbelt to reach the southern hemisphere. While these results confirm the qualitative correctness of earlier estimates<sup>4,10</sup>, our simulation, using the more probable value of  $w_{\text{eff}}$ , predicts dry deposition that is significantly higher than earlier estimates and export from the continent that is at the lower bound of past estimates. In particular, a realistic meteorological model transports much less to Europe than does a model driven by seasonal mean winds<sup>10</sup>.

The upper limit for  $w_{\text{eff}}$  [ $1.0 \text{ cm s}^{-1}$ ] requires that over half of the  $\text{NO}_y$  exist as  $\text{HNO}_3$  and results in complete domination of the nitrogen budget by dry deposition. The resulting surface concentrations of  $\text{NO}_y$  are much less than observed, and an

unrealistically high value of  $N$  is needed to even approach the observed deposition in acid rain.

Simulations of yearly precipitation and its nitrogen content for  $w_{\text{eff}}$  set at 0.3 and  $0.1 \text{ cm s}^{-1}$  over land are compared in Table 2 with observations from a selection of United States and Canadian stations<sup>21–23,25</sup>. The spatial pattern and most of the individual stations are in good agreement with observations. The model's dryness in the mid-west leads to a slight underestimate of acid rain there and similarly the excess precipitation in the far north-east leads to a slight overestimate. The three stations that are significantly higher than observed (Ithaca, Whiteface and Brookhaven) are all relatively unpolluted sites near regions of high emissions for which the model's 265-km grid resolution is not sufficient. The last five stations in Table 2 represent export to the North Atlantic. The predicted export to Bermuda is a little low but it is quite good for the Maritimes and northern Quebec.

While the model's grid resolution is too coarse to resolve areas of low emission embedded in or near regions of significant combustion, it is adequate to resolve a regional source embedded in North America (cross-hatched area in Fig. 1). We shut off 85% of the combustion nitrogen source and leave it on in 16 grid-boxes in the northeastern United States and eastern Canada. The simulation is then run for 15 months with our most realistic estimate for  $w_{\text{eff}}$  ( $0.3 \text{ cm s}^{-1}$ ) and its companion value  $N = 9$ . By comparing the deposition over the northeastern United States and eastern Canada from this simulation with that from the previous one using the complete combustion source, we can determine the relative importance of regional and distant sources to acid rain, dry deposition and total deposition of acid nitrogen in the region. The various contributions are shown in Table 3. Acid rain in the north-east is dominated by emissions transported from distant sources, while dry deposition comes overwhelmingly from emissions in the north-east. Over half of the total accumulation of acidic nitrogen is a result of local and regional emissions. While acid rain and dry deposition are comparable over the region as a whole, dry deposition dominates in the northeastern United States and the populated regions of eastern Canada. Only in northern Canada far from major sources, do acid rain and transport from distant sources dominate.

**Table 3** Nitrogen deposition in the northeastern United States and eastern Canada

| Emission source | Acid precipitation<br>( $\text{Tg(N)yr}^{-1}$ ) | Dry deposition<br>( $\text{Tg(N)yr}^{-1}$ ) | Total deposition<br>( $\text{Tg(N)yr}^{-1}$ ) |
|-----------------|-------------------------------------------------|---------------------------------------------|-----------------------------------------------|
| Regional        | 0.28                                            | 0.48                                        | 0.76                                          |
| Distant         | 0.50                                            | 0.13                                        | 0.63                                          |



Given these results, we conclude that the current acid rain networks in the United States and Canada measure less than half of the acidic nitrogen that is accumulating in the environment, and that the current monitoring programme in the north-eastern United States and eastern Canada exaggerates the role of transport from the mid-west, south-east and Ohio Valley and underestimates the importance of local and regional emissions. Given this apparent major role for dry deposition, it is important that we determine its impact on the acidification of the environment.

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## Six-coordinated silicon in glasses

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It has been accepted since 1932 that the building block of silicate glasses consists of silicon tetrahedrally coordinated to 4 oxygen atoms<sup>1</sup>. Although the existence of 6-coordinated silicon in a few crystalline materials, such as stishovite, and  $\text{SiP}_2\text{O}_7$  is known<sup>2,3</sup>, the presence of  $(\text{SiO}_6)$  units has not yet been observed experimentally in glasses. Recent X-ray diffraction and magic-angle-spinning (MAS) nuclear magnetic resonance investigations<sup>4,5</sup> of  $\text{SiO}_2$ - $\text{P}_2\text{O}_5$  glasses found only 4-coordinated silicon although  $\text{SiP}_2\text{O}_7$  was produced by devitrification. Raman studies<sup>6</sup> of  $\text{Na}_2\text{O}$ - $\text{SiO}_2$ - $\text{P}_2\text{O}_5$  glasses were interpreted as indicating the presence of a structural unit containing Si-O-P bonding but otherwise unidentifiable. We have applied MAS nuclear magnetic resonance spectroscopy to similar glasses and here we report the first observation of  $[\text{SiO}_6]$  units in glasses.

Sodium disilicate base glasses NSP1-NSP4, with different amounts of phosphorus as given in Table 1, were prepared from mixtures of analytical grade diammonium hydrogen phosphate, silica and sodium carbonate. The glass batch was preheated at 200 °C for 1.5 h to volatilize  $\text{NH}_3$  and  $\text{H}_2\text{O}$  and then melted in alumina crucibles at 1,575 °C for 4 hours (NSP1) or 1,100 °C for 2.5 h (NSP2, 3, 4). All samples were splat-cooled between two graphite coated steel plates at room temperature and were subsequently stored in a desiccator.  $\text{MnCO}_3$  at 0.05 mol % was

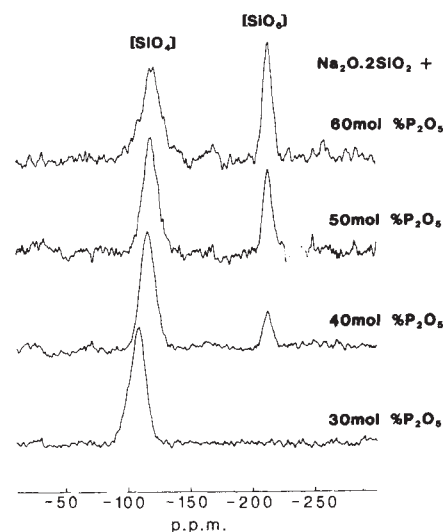


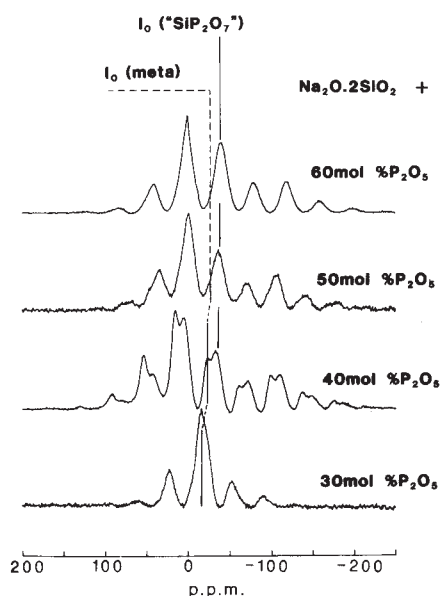
Fig. 1 MAS-NMR spectra of  $^{29}\text{Si}$  in sodium disilicate glasses containing 30, 40, 50 and 60 mole %  $\text{P}_2\text{O}_5$ . Approximately 0.4 g of sample were used in Delrin rotors which were spun at 3.0-3.5 kHz.

added to each batch to reduce the  $^{29}\text{Si}$  relaxation time, since this is typically 10 min in undoped samples. Control experiments on undoped samples showed that there was no difference in relative peak intensities due to small additions of  $\text{MnCO}_3$ . All MAS nuclear magnetic resonance (NMR) spectra were recorded on a Bruker MSL 360 spectrometer operating at  $\sim 71.5$  MHz for  $^{29}\text{Si}$  and 145.8 MHz for  $^{31}\text{P}$  nuclei. The delays between pulses were chosen by increasing the relaxation delay until the relative intensities from different species did not alter. Typically 2- $\mu\text{s}$  ( $\sim \pi/6$ ) pulses with a 60-s delay were used for acquiring  $^{29}\text{Si}$  spectra and 4  $\mu\text{s}$  ( $\sim \pi/2$ ) pulses with 1 s delay for  $^{31}\text{P}$ . Chemical shifts were measured relative to tetramethylsilane, 85%  $\text{H}_3\text{PO}_4$  and dilute aqueous NaCl for  $^{29}\text{Si}$ ,  $^{31}\text{P}$  and  $^{23}\text{Na}$  respectively. As some contamination from the alumina crucibles was anticipated,  $^{27}\text{Al}$  spectra were also acquired with aqueous  $\text{Al}(\text{NO}_3)_3$  as reference. From the size of the  $^{27}\text{Al}$  signal and also from dispersive X-ray energy analysis, the level of contamination was estimated to be 2 mol %. Glasses NSP2-4 were transparent and X-ray amorphous. However, in NSP1, sporadic nucleation was observed but the clear portions of the glass selected for NMR experiments were shown to be X-ray amorphous within the detection limit of X-ray diffraction, that is significantly less than 5% crystalline. The spectra recorded for  $^{29}\text{Si}$  and  $^{31}\text{P}$  are illustrated in Figs 1 and 2 and the isotropic peak positions, halfwidths and relative intensities are recorded in Table 1.

We have recently reported<sup>7</sup> that the addition of small amounts (1-5 mol %) of  $\text{P}_2\text{O}_5$  to alkali disilicate glasses results in the scavenging of modifier cations by phosphorus to give orthophosphate  $\text{M}_3\text{PO}_4$  and pyrophosphate  $\text{M}_4\text{P}_2\text{O}_7$  units accompanied by polymerization of the silicate network, that is, elimination of non-bridging oxygens and conversion of  $\text{Q}^3 \text{Si}(\text{OSi})_3\text{O}^-$  to  $\text{Q}^4 \text{Si}(\text{OSi})_4$  silicons. The phosphorus thus appears to play no part in the glass network at these concentrations. Above  $\sim 10$  mole %  $\text{P}_2\text{O}_5$ , glass formation is difficult because of high liquidus temperatures but becomes easier beyond 30 mol %  $\text{P}_2\text{O}_5$ . Up to 30 mol %  $\text{P}_2\text{O}_5$  we observe increasing polymerization of the phosphate groups, first to give pyrophosphate dimers, then metaphosphate chains and the silicon spectra show the presence of  $\text{Q}^4$  only. However, the MAS spectra obtained from glasses of  $>30$  mol %  $\text{P}_2\text{O}_5$  content are now most unusual. In the 30 mol %  $\text{P}_2\text{O}_5$  glass (NSP1) a single peak is observed in the  $^{29}\text{Si}$  spectrum at  $-109$  p.p.m., characteristic of pure  $\text{Q}^4$  as in vitreous  $\text{SiO}_2$  undiluted by other species. The  $^{31}\text{P}$  spectrum also contains a single resonance (plus associated side bands) at  $-16$  p.p.m. which is characteristic of metaphosphate ( $\text{NaPO}_3$ )<sub>n</sub>.

**Table 1** Composition and spectral properties of sodium disilicate base glasses with differing phosphorus contents

| Nominal compositions<br>(mol %) |                   |                  |                               | Spectral parameters (p.p.m.) ±0.05 |             |                   |      |                  |      |                  |              |                                           |
|---------------------------------|-------------------|------------------|-------------------------------|------------------------------------|-------------|-------------------|------|------------------|------|------------------|--------------|-------------------------------------------|
| Sample                          | Na <sub>2</sub> O | SiO <sub>2</sub> | P <sub>2</sub> O <sub>5</sub> | <sup>29</sup> Si                   |             | <sup>31</sup> P   |      | <sup>23</sup> Na |      | <sup>27</sup> Al |              | SiO <sub>6</sub> :Q <sup>4</sup><br>ratio |
|                                 |                   |                  |                               | Chemical<br>shift                  | FWHM        | Chemical<br>shift | FWHM | Peak<br>posn     | FWHM | Peak<br>posn     | FWHM         |                                           |
| NSP1                            | 23.34             | 46.66            | 30.0                          | −109.0                             | 14.0        | −16.0             | 16.5 | −17.5            | 32.0 | +42.5<br>−14.4   | 21.1<br>17.4 | 0                                         |
| NSP2                            | 20.0              | 40.0             | 40.0                          | −116.6<br>−212.6                   | 15.0<br>9.1 | −22.9<br>−32.7    |      | −14.5            | 27.2 | −17.5            | 12.4         | 0.15                                      |
| NSP3                            | 16.67             | 33.33            | 50.0                          | −118.4<br>−213.1                   | 15.5<br>9.0 | −36.5             | 17.0 | −16.8            | 26.0 | −18.5            | 12.4         | 0.44                                      |
| NSP4                            | 13.33             | 26.67            | 60.0                          | −119.9<br>−212.8                   | 18.0<br>9.0 | −39.5             | 17.0 | −17.7            | 26.0 | −19.0            | 12.2         | 0.59                                      |



**Fig. 2** MAS-NMR spectra of <sup>31</sup>P in sodium disilicate glasses containing 30, 40, 50 and 60 mole % P<sub>2</sub>O<sub>5</sub>. Approximately 0.4 g of sample were contained in alumina rotors and spun at speeds up to 5.0 kHz. I<sub>0</sub> (meta)—isotropic peak arising from metaphosphate-like groups. I<sub>0</sub> ("SiP<sub>2</sub>O<sub>7</sub>")—isotropic peak arising from <sup>31</sup>P in an SiP<sub>2</sub>O<sub>7</sub>-like environment. All other peaks are spinning side bands.

chains. This agrees with a scavenging of sodium ions by phosphate, the compositional ratio Na/P being 0.78 in the glass and 1.0 in metaphosphate.

When further additions of P<sub>2</sub>O<sub>5</sub> are made (NSP2, 3, 4) an additional resonance appears in the silicon spectrum at -213 p.p.m. The intensity of this peak, relative to that of Q<sup>4</sup>, increases with increasing P<sub>2</sub>O<sub>5</sub> content. Several authors<sup>5,8,9</sup> report similar chemical shifts for <sup>29</sup>Si when 6-coordinated, as in cubic SiP<sub>2</sub>O<sub>7</sub> (-214 p.p.m.) and Si<sub>3</sub>O(PO<sub>4</sub>)<sub>6</sub> (-217 p.p.m.). The position of the "Q<sup>4</sup>" silicon resonance shifts from that observed in the 30 mol % P<sub>2</sub>O<sub>5</sub> material. It is now found at -116.5 (40%), -118.4 (50%), or -119.9 (60%). This shift may indicate some Si-O-P bond formation, the increased electronegativity of phosphorus leading to a more negative shift. There can be six crystallographically distinct sites in SiP<sub>2</sub>O<sub>7</sub> (ref. 3). If these glasses contain structural units similar to those in SiP<sub>2</sub>O<sub>7</sub> then the Si resonance of [SiO<sub>6</sub>] might be expected to be multiple or broadened. This is not so—the resonance in fact being relatively narrow (9 p.p.m.) compared with vitreous silica (12 p.p.m.) or the Q<sup>4</sup> units in these glasses (16 p.p.m.). This may indicate that in these glasses the [SiO<sub>6</sub>] unit is in fact less distorted than in the crystalline material.

The <sup>31</sup>P spectra for these glasses show, in addition to the metaphosphate peak at -16 p.p.m., the appearance of a new peak with increasing P<sub>2</sub>O<sub>5</sub> content. The chemical shift of this peak changes from -32.7 to -39.5 on going from 40 to 60 mol % P<sub>2</sub>O<sub>5</sub>. In the latter glass NSP4, this is the only peak present. We presume that this chemical shift corresponds to phosphorus in an environment similar to that in SiP<sub>2</sub>O<sub>7</sub> although the reported

shifts in the crystalline material are from -46 to -54 p.p.m. (ref. 9). It should be remembered that there is also some sodium in the glass. There is little change in the chemical shift of <sup>23</sup>Na with P<sub>2</sub>O<sub>5</sub> content. However, the sodium must play an important role in stabilizing a network comprising [SiO<sub>4</sub>], [PO<sub>4</sub>] and [SiO<sub>6</sub>]. The environment of the Al<sup>3+</sup> impurity in these glasses also changes with increasing P<sub>2</sub>O<sub>5</sub> content. In the 30 mol % glass, NSP1, two peaks are observed, one at +42.5 p.p.m. corresponding to Al<sup>3+</sup> tetrahedrally coordinated via oxygens to phosphorus or possibly silicon and one at -14 p.p.m. which we assign to Al<sup>3+</sup> octahedrally coordinated via oxygens to phosphorus. This latter peak is the only one observed in NSP2, 3 and 4. This implies that Al<sup>3+</sup> is substituting for Si<sup>4+</sup> and thus shows a similar switch from 4- to 6-coordination as the concentration of phosphorus is increased.

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# A clathrate hydrate of carbon monoxide

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Since the mid-1950s it has been known that most simple clathrate hydrates belong to one of two cubic crystal forms known as type I and II<sup>1-3</sup>. Only recently it was established that structure II hydrates are formed by small guests such as O<sub>2</sub>, N<sub>2</sub>, Kr and Ar<sup>4</sup> as well as molecules larger than ~0.58 nm, with molecules of intermediate size forming type I hydrate. The existence of a hydrate of carbon monoxide, a molecule similar in size to O<sub>2</sub> and N<sub>2</sub>, has been proposed since ~1960<sup>5,6</sup>. This hydrate is thought to play an important role in comets and the outer planets in the Solar System<sup>7,8</sup>. We have now prepared carbon monoxide hydrate, and demonstrate its clathrate hydrate nature from dielectric and <sup>13</sup>C NMR (nuclear magnetic resonance) measurements. Unexpectedly, X-ray powder diffraction shows that, unlike hydrates of O<sub>2</sub> and N<sub>2</sub>, carbon monoxide has a type I cubic structure with a lattice parameter of 1.188 nm.

The century-old puzzle of the nature of gas hydrates was solved in the mid-1950s, mainly by the X-ray diffraction studies of von Stackelberg and his students<sup>1-3</sup>. They found that all thirty gas hydrates which they examined were water clathrates of one or the other of the two distinct cubic crystal structures, types I and II. Relatively small molecules (≤5.7 Å maximum van der Waals diameter) formed structure I hydrates, which contained six to eight water molecules per molecule of guest, while structure II was formed by larger molecules and contained ~17 water molecules per guest molecule. Until very recently there seemed no reason to doubt this general rule. By 1984 some 110 simple clathrate hydrates were known<sup>9,10</sup>. About 40 of these were considered to be type I and the remainder type II on the basis of X-ray diffraction or compositional studies or both.

For the smallest gas-hydrate-forming molecules (Ar, Kr, O<sub>2</sub>, N<sub>2</sub>, CH<sub>4</sub> in order of increasing size) the estimated hydration numbers are all about 6 (ref. 9). Presumably because the hydrates of all these molecules are only stable under elevated pressures under normal conditions (the dissociation pressure at 0 °C of the hydrates of Ar, Kr, O<sub>2</sub>, N<sub>2</sub> and CH<sub>4</sub> are 96, 15, 120, 161 and 26 atm, respectively), no diffraction studies were made to confirm the assumption of structure I. Such studies may, however, be made on hydrate samples which are cooled sufficiently to prevent hydrate dissociation during the accumulation of diffraction data. Recently<sup>4</sup>, and unexpectedly, low-temperature studies of the hydrates of argon and krypton showed them to be structure II, while structure I was confirmed for methane hydrate. More recently the hydrates formed by O<sub>2</sub> and N<sub>2</sub><sup>5,6</sup>, molecules intermediate in size between krypton and methane, have been found<sup>11,12</sup> to be structure II.

Here we give the results of studies of the hydrate formed by carbon monoxide, a molecule similar in size to O<sub>2</sub> and N<sub>2</sub>. As far as we know, this hydrate has never been reported before, although its formation has been anticipated at least since 1960<sup>5,6</sup> and CO is often assumed to be one of the guest species in mixed gas hydrates thought to be present in comets. For CO hydrate dissociation pressures comparable to the high dissociation pressures of N<sub>2</sub> and O<sub>2</sub> hydrates (141 and 120 bars, respectively at 0 °C<sup>5,6</sup>) are likely; Miller has recently predicted<sup>7</sup> formation of a structure II CO hydrate with a dissociation pressure of about 135 bars at 0 °C.

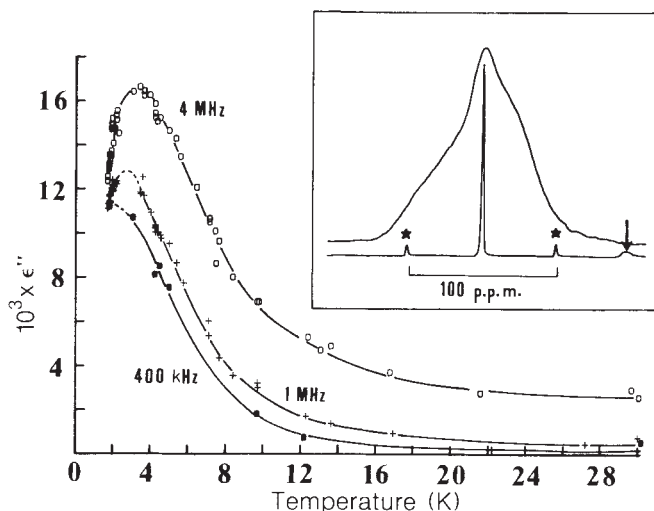


Fig. 1 Low-temperature dielectric absorption of carbon monoxide hydrate. Inset: <sup>13</sup>C NMR spectrum of carbon monoxide hydrate at 153 K (top); same for spinning sample at 153 K (bottom); spinning side bands are starred, arrow marks Delrin peak at 88.1 p.p.m.

Samples of carbon monoxide hydrate were initially prepared, by the method already described for argon and krypton hydrates<sup>4</sup>, in a pressure vessel from powdered ice at about -30 °C and gaseous carbon monoxide at a pressure of about 173 bars (2,500 psig). The CO was Matheson research quality grade. Hydrate formation, followed by the fall in pressure, was complete after two or three days of reaction, during which the sample was subjected to the grinding action of several cylindrical steel rods present in the slowly rotating pressure vessel. Hydrate samples were then prepared for dielectric, NMR and X-ray powder diffraction measurements. For the NMR study a sample enriched to 50% in <sup>13</sup>CO was used, and because of a limited amount of material, this hydrate sample was prepared at somewhat lower pressure and temperature, 88 bars at 240 K.

The dissociation pressure of CO hydrate at 0 °C corresponding to the three-phase hydrate-liquid water-gas equilibrium was determined, after equilibrating the pressure vessel in an ice bath, by systematically reducing the pressure in small steps until eventually the pressure was found to repeatedly rise to the same constant value, 128 bars.

In an attempt to define the dipole reorientation rates of enclathrated CO molecules, dielectric measurements below 100 K were made at audio and low radio frequencies essentially as already described<sup>13</sup>. The hydrate sample was rapidly pressed into a flat disk in a pre-cooled die and mounted between the parallel electrodes of a three-terminal cell mounted in an Andonian Associates liquid helium cryostat. As the electric dipole moment is very small (about 0.13 Debye) the measuring apparatus and frequencies were chosen to resolve best the small dielectric losses from losses inherent in the cell and bridges used<sup>14</sup>. With the cell constant estimated from the sample dimensions, the measured permittivities are expected to be systematically low because of sample porosity and surface roughness.

Results of low-temperature dielectric loss factor measurements at a number of fixed frequencies are shown in Fig. 1a. There is a region of small, very broad dielectric absorption and dispersion at the lowest temperatures which must be ascribed to the reorientation of encaged CO molecules. In solid CO much narrower dielectric absorption corresponding to a single relaxation time occurs at a much higher temperature (for example, maximum loss at 10 kHz is found at 32.4 K<sup>15</sup>). The corresponding barrier to reorientation is in solid CO 6.1 kJ mol<sup>-1</sup><sup>15</sup>.

The great width of the dielectric absorption by guest molecules quite generally observed in clathrate hydrates at low tem-

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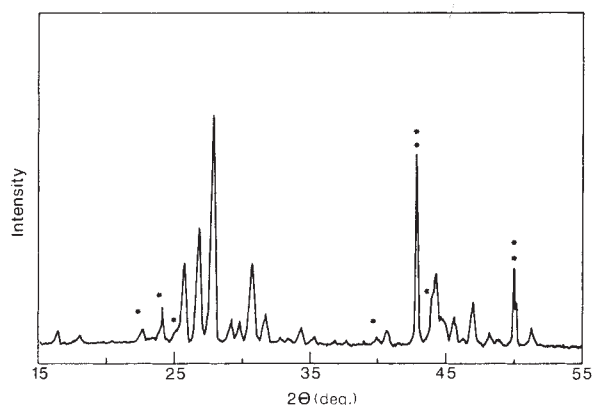


Fig. 2 X-ray powder diffraction pattern of carbon monoxide hydrate. Starred lines are due to ice Ih, double starred lines are due to the sample holder.

peratures<sup>10</sup> arises from the frozen-in orientational disorder of the water molecules of the lattice. For enclathrated CO the average barrier to reorientation (about 0.14 kJ mol<sup>-1</sup>) is comparable to that found<sup>14</sup> for H<sub>2</sub>S in its hydrate. CO is perhaps the most rotationally mobile dipolar guest species yet found. The <sup>13</sup>C NMR spectrum of CO hydrate (Fig. 1, inset) was obtained at 45.3 MHz on a Bruker CXP-180 NMR spectrometer equipped with a Chemagnetics probe under CP/MAS conditions at -120 °C. Unfortunately only a single line was observed at 184 p.p.m. with a line width of 72 Hz. For a number of hydrates, resonances of guest molecules in different cages are shifted with respect to each other. This allows a determination of hydrate structure type, for example, for methane in structure I hydrate, the CH<sub>4</sub> small-cage resonance is downfield by 2.4 p.p.m. from the large-cage resonance. But the fact that a <sup>13</sup>C spectrum was obtained under <sup>13</sup>C-<sup>1</sup>H cross-polarization conditions confirms 'solid-like' behaviour for the CO molecule, that is, there are <sup>13</sup>C-<sup>1</sup>H nuclear dipolar interactions which should exist only for molecules encaged in the hydrate lattice.

The <sup>13</sup>C line-shape obtained for a static sample at 153 K shows some asymmetry which is in the opposite sense to the asymmetry in the low-temperature <sup>13</sup>C NMR line-shape of solid CO itself<sup>16</sup>. This may reflect partial averaging of the <sup>13</sup>C nuclear shielding tensor due to motions in which the molecular long axis is constrained to lie near to the equatorial plane of the type I hydrate large cage, as was previously observed for CO<sub>2</sub> and COS guest molecules at higher temperatures<sup>17,18</sup>.

X-ray diffraction patterns were obtained for two different hydrate samples with Cu Kα radiation on a Rigaku θ-θ powder diffractometer equipped with an Anton-Paar low-temperature

camera. Figure 2 shows the pattern obtained at 90 K for the sample prepared at 173 bars, -30 °C. The pattern was indexed in terms of von Stackelberg's cubic type I hydrate, space group *Pm3n*. A least squares fit to 17 indexed reflections gave a unit cell size of 1.1877(4) nm (Table 1). The sample prepared for the NMR study at 88 bars, -33 °C, gave a similar pattern, although a greater proportion of Ice Ih was present.

That carbon monoxide forms a type I hydrate is rather surprising, as the structure II hydrate former N<sub>2</sub> is very similar to carbon monoxide in size and electronic structure. Lunine and Stevenson<sup>8</sup> have attempted to calculate the dissociation pressure of CO hydrate from the classical Lennard-Jones-Devonshire cell model with interaction parameters not specified but apparently modified from those of nitrogen hydrate. They find the dissociation pressure of structure I CO hydrate to be 20% less than that of structure II.

Interaction parameters obtained from second virial coefficients and viscosities of the gases are not much different for O<sub>2</sub>, N<sub>2</sub> and CO<sup>19</sup>. It is likely that the preference of CO for structure I is related to the specifics of the guest-water interactions in the two structures. CO differs from O<sub>2</sub> and N<sub>2</sub> in being (slightly) dipolar and in having a higher quadrupole moment and molecular polarizability. These factors may be important enough to make the heat of engagement in the 14-hedral structure I cavities significantly more negative than in the more symmetric 12-hedral cages if the resultant electric fields of the water molecules are greater in the 14-hedra, but a large effect of this kind is not anticipated.

It is not clear why the dissociation pressure of CO hydrate should be intermediate between the dissociation pressures of O<sub>2</sub> and N<sub>2</sub> hydrates if its structure is different.

The formation of structure II hydrates by four of the smallest gas-hydrate forming molecules (Ar, Kr, O<sub>2</sub>, N<sub>2</sub>) is consistent with preferred occupancy of the small pentagonal dodecahedral cages: these make up two thirds of the cages in the type II lattice but only one quarter of those in type I. Full occupancy of the 12- and 16-hedral cages of structure II gives five and two-thirds molecules of water per molecule of guest, a ratio not much different from the five and three-quarters given by complete occupancy of the 14- and 12-hedral cages of structure I, but greatly different from the ratio of 17 found for the 'usual' structure II hydrates formed by molecules which are too large to enter any but the 16-hedral cages. It would be difficult to distinguish between structures I and II of small molecules on the basis of the composition; although II is capable of accommodating slightly more guest molecules, the minimum degree of cage occupancy required for hydrate stability is appreciably less for II than for I<sup>5,6</sup>. Structure I is apparently more stable with molecules which fit best into the intermediate-sized 14-hedral cages. CO apparently falls into this category.

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Table 1 X-ray powder diffraction data

| Miller indices<br><i>hkl</i> | 2θ (deg.) | <i>d</i> (obs.) | <i>d</i> (calc.) |
|------------------------------|-----------|-----------------|------------------|
| 012                          | 16.675    | 5.316           | 5.311            |
| 112                          | 18.306    | 4.846           | 4.849            |
| 222                          | 25.980    | 3.429           | 3.429            |
| 023                          | 27.055    | 3.296           | 3.294            |
| 123                          | 28.115    | 3.174           | 3.174            |
| 044                          | 30.079    | 2.970           | 2.969            |
| 014                          | 30.941    | 2.890           | 2.880            |
| 033; 114                     | 31.989    | 2.798           | 2.799            |
| 124                          | 34.643    | 2.589           | 2.592            |
| 233                          | 25.539    | 2.526           | 2.532            |
| 025; 234                     | 40.928    | 2.205           | 2.206            |
| 035; 334                     | 44.571    | 2.033           | 2.037            |
| 135                          | 45.085    | 2.011           | 2.010            |
| 016                          | 45.876    | 1.978           | 1.980            |
| 116; 235                     | 47.207    | 1.925           | 1.927            |
| 026                          | 48.474    | 1.878           | 1.878            |
| 036; 245                     | 51.480    | 1.774           | 1.771            |

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## Floristic diversity in a model system using experimental microcosms

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Most investigations of floristic diversity have involved studies<sup>1-15</sup> of natural vegetation. Progress using these approaches has been limited because some potentially important factors are not amenable to precise field measurement or manipulation. Here we describe an alternative research strategy in which communities were allowed to develop in turf microcosms providing factorial combinations of soil heterogeneity, grazing and mycorrhizal infection, all of which are capable in theory<sup>6,10,13,16-19</sup> of promoting diversity. Both grazing and mycorrhizas increased diversity markedly by raising the biomass of the subordinate species relative to that of the canopy dominant. The effect of grazing is shown to be due to the differential sensitivity of the canopy dominant to defoliation. Export of assimilate from canopy to subordinate species through a common mycelial network is likely, together with enhancement of mineral nutrient capture, to be involved in the beneficial effect of mycorrhizas. No major effects of soil heterogeneity upon diversity were detected.

In the humped-back model<sup>1,5,6</sup> explaining patterns of variation in species richness in herbaceous vegetation, it is suggested that the potential for high diversity is restricted to a corridor which, in the British Isles, corresponds to the range 350–750 g dry weight m<sup>-2</sup> in seasonal maximum in standing crop plus litter. Below this range, diversity is suppressed because the severity of habitat conditions excludes all but a few specialized species whereas, above it, diversity falls with increasing biomass and dominance by a small number of robust species. Although this model defines in general terms the circumstances which are not conducive to high diversity it does not identify the mechanisms which permit species coexistence within the intermediate or 'corridor' communities. Hypotheses which seek to explain coexistence form a spectrum, the extremes of which correspond to the so-called equilibrium<sup>16,17</sup> and non-equilibrium<sup>10,13,18</sup> models. The former propose that species coexist by spatial or temporal partitioning of resources or opportunities for regeneration whereas the latter explain diversity as a result of a dynamic micro-successional mosaic generated by vegetation disturbance.

Our own field observations and experiments<sup>1,7,9,20-22</sup> in highly contrasted habitats and vegetation types of high diversity strongly suggest that both equilibrium and non-equilibrium mechanisms operate in nature and we suspect that often the two function together in the same community. However, the protracted nature of the debate about the causes of high floristic diversity is not primarily due to the probability that both arguments have some validity. The main obstacles in current efforts to resolve this problem are the difficulties encountered in applying rigorous experimental tests using natural plant communities. Here we present the results of an experiment designed to circumvent some of these difficulties by examining the effect of specific factors on diversity in laboratory microcosms. The three factors chosen for study (soil heterogeneity, grazing and mycorrhizal infection) have all been implicated in diversity theories by earlier investigators<sup>6,10,13,16-19</sup> but the effects of each are exceedingly difficult to quantify in natural vegetation.

Each of the 40 microcosms in our experiment had external dimensions of 23 × 23 × 10 cm and contained 3,113 cm<sup>3</sup> of nutrient-poor calcareous sand which had been sterilized by  $\gamma$ -irradiation. Treatments involving spatial heterogeneity, mycorrhizal infection and grazing were provided in factorial

**Table 1** Influence of soil heterogeneity, grazing and mycorrhizal infection on floristic diversity of turf microcosms

| Soil heterogeneity | Grazing | VA mycorrhizas | Mean no. species per microcosm | Mean Shannon diversity index |
|--------------------|---------|----------------|--------------------------------|------------------------------|
| –                  | –       | –              | 15.2 (1.0)                     | 0.23 (0.07)                  |
| +                  | –       | –              | 15.8 (2.8)                     | 0.20 (0.03)                  |
| –                  | +       | –              | 14.4 (1.7)                     | 0.59 (0.14)                  |
| –                  | –       | +              | 15.0 (2.5)                     | 0.43 (0.15)                  |
| +                  | +       | –              | 16.0 (2.2)                     | 0.61 (0.13)                  |
| +                  | –       | +              | 16.2 (1.8)                     | 0.44 (0.23)                  |
| –                  | +       | +              | 14.2 (2.0)                     | 0.68 (0.16)                  |
| +                  | +       | +              | 17.2 (1.0)                     | 0.78 (0.14)                  |

Diversity indices are based on shoot dry weights measured at the end of the experiment. Each value is the mean of five replicates. Confidence limits (95%) are presented in brackets.

**Table 2** Size of seedlings of *Centaurea erythraea* in the presence and absence of VA mycorrhizas

|                               | Number of seedlings: |       |
|-------------------------------|----------------------|-------|
|                               | >5 mm                | <5 mm |
| With mycorrhizal infection    | 147                  | 485   |
| Without mycorrhizal infection | 0                    | 814   |

Rosettes were measured 2 months after seed was sown.

combinations, each of which was replicated five times. Throughout the experiment, mineral nutrients were supplied as aliquots of dilute Rorison solution (5%) with occasional pulses of full strength Rorison solution. All microcosms were independent of each other and were maintained in a randomized block in a growth room providing a temperature of 17 °C over a 16-hour day with a night temperature of 12 °C. Vesicular-arbuscular (VA) mycorrhizal fungi were introduced to half the microcosms by an initial sowing, using a rectangular grid, of 16 seedlings of *Festuca ovina* which had been infected by a natural inoculum of washed root pieces in which *Glomus constrictum* was the dominant sporulating mycorrhizal fungus. The same number of uninfected seedlings, which had been raised in sand containing sterilized root pieces, was planted in the remaining microcosms. The experiment extended over twelve months and involved an initial germination phase in which a standardized seed inoculum of twenty herbaceous species was introduced to each microcosm. Most of the plants used in the experiment were common herbaceous species which frequently occur together in grasslands on shallow calcareous soils. Each seed was positioned using random coordinates, redistributed under simulated rainfall, then allowed to germinate under humid conditions. Soil heterogeneity involved 'crevices', 'exposed rocks', 'buried rocks', 'cups' (without drainage) and 'talus', of standardized dimensions and number, manufactured from plastic, and occupying different randomized positions within each microcosm. The main effect of this treatment was to produce heterogeneity in rooting depth and soil moisture status without alteration of the total volume of sand provided.

After the establishment phase the grazing treatment was applied at intervals of 3 weeks to half the microcosms; this consisted of bouts of defoliation by scissors, randomized with respect to bite size and position, in each of which 10% of the turf was grazed lightly (to 25 mm) and 10% closely (to 5 mm). The position of each surviving seedling was recorded at intervals during the experiment and after 12 months the shoot of each plant was harvested individually and its dry weight determined.

Table 1 is based upon diversity indices calculated for each microcosm using the total shoot biomass of each species at the end of the experiment. These data show that whereas soil heterogeneity did not exert a significant effect upon floristic



**Table 3** The effect of grazing and mycorrhizal infection on yield (mg) per plant in species grown together in turf microcosms for one year

|                               | Ungrazed/no<br>mycorrhizal<br>infection | Ungrazed/<br>mycorrhizal<br>infection | Grazed/no<br>mycorrhizal<br>infection | Grazed/<br>mycorrhizal<br>infection | Analysis of variance    |                       |             |
|-------------------------------|-----------------------------------------|---------------------------------------|---------------------------------------|-------------------------------------|-------------------------|-----------------------|-------------|
|                               |                                         |                                       |                                       |                                     | Effects of<br>infection | Effects of<br>grazing | Interaction |
| <b>Grasses</b>                |                                         |                                       |                                       |                                     |                         |                       |             |
| <i>Anthoxanthum odoratum</i>  | 5.02                                    | 5.40                                  | 4.19                                  | 6.46                                | —                       | —                     | —           |
| <i>Briza media</i>            | 13.55                                   | 54.26                                 | 19.47                                 | 23.76                               | *                       | NS                    | NS          |
| <i>Dactylis glomerata</i>     | 160.19                                  | 286.84                                | 180.63                                | 240.91                              | NS                      | NS                    | NS          |
| <i>Festuca ovina</i> +        | 922.68                                  | 609.43                                | 241.42                                | 141.83                              | ***                     | ***                   | NS          |
| <i>Festuca ovina</i>          | 24.14                                   | 22.02                                 | 16.78                                 | 9.31                                | *                       | ***                   | NS          |
| <i>Festuca rubra</i>          | 27.67                                   | 25.37                                 | 31.59                                 | 23.09                               | *                       | NS                    | NS          |
| <i>Poa pratensis</i>          | 15.07                                   | 19.34                                 | 16.95                                 | 15.17                               | NS                      | NS                    | NS          |
| <b>Forbs</b>                  |                                         |                                       |                                       |                                     |                         |                       |             |
| <i>Arabis hirsuta</i>         | 0.26                                    | 0.13                                  | 0.13                                  | 0.14                                | —                       | —                     | —           |
| <i>Campanula rotundifolia</i> | 0.73                                    | 4.20                                  | 3.31                                  | 10.68                               | **                      | ***                   | NS          |
| <i>Centaurea nigra</i>        | 1.70                                    | 10.90                                 | 2.78                                  | 15.46                               | ***                     | **                    | NS          |
| <i>Centaureium erythraea</i>  | 0.23                                    | 7.08                                  | 0.02                                  | 5.18                                | —                       | —                     | —           |
| <i>Galium verum</i>           | 1.87                                    | 9.39                                  | 4.46                                  | 7.26                                | **                      | *                     | NS          |
| <i>Hieracium pilosella</i>    | 0.93                                    | 7.63                                  | 3.34                                  | 8.73                                | ***                     | NS                    | NS          |
| <i>Leontodon hispidus</i>     | 0.83                                    | 3.72                                  | 2.16                                  | 15.99                               | **                      | *                     | NS          |
| <i>Plantago lanceolata</i>    | 3.62                                    | 33.96                                 | 4.29                                  | 24.07                               | ***                     | NS                    | *           |
| <i>Rumex acetosa</i>          | 9.72                                    | 8.77                                  | 7.62                                  | 11.14                               | NS                      | NS                    | NS          |
| <i>Sanguisorba minor</i>      | 5.06                                    | 17.14                                 | 5.93                                  | 22.68                               | ***                     | NS                    | NS          |
| <i>Scabiosa columbaria</i>    | 2.46                                    | 10.19                                 | 9.84                                  | 31.97                               | ***                     | ***                   | NS          |
| <i>Silene nutans</i>          | 16.85                                   | 44.89                                 | 40.79                                 | 79.08                               | ***                     | ***                   | NS          |

The analysis of variance is based on mean seedling shoot dry weight for each of the 18 species in each of the 40 microcosms. For all species the ANOVA was performed on log transformed data with the exception of *Festuca rubra* and *Poa pratensis* (untransformed) and *Festuca ovina* + (square root transformed). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; NS, no statistical significance; —, insufficient survivors for ANOVA. *Festuca ovina* plants are separated into those which were introduced as small seedlings (+), and those sown from seed. Two additional species, *Juncus bufonius* and *Veronica arvensis*, had no survivors at the end of the experiment.

diversity both grazing and mycorrhizal infection were strongly influential.

The effect of the grazing treatment was exerted through the relatively greater sensitivity to defoliation of the canopy dominant *Festuca ovina*. This is evident in Table 3 which shows that the mean shoot weight of individuals of *Festuca ovina* fell significantly, whereas those of shorter subordinate species, like *Centaurea nigra* and *Silene nutans*, were stimulated by the grazing treatment. Such results of random simulated grazing show that it is not necessary to invoke any sensory discrimination between species by the grazing animal to obtain a promotion of diversity.

The greater diversity associated with mycorrhizas was manifested in part through the survival of seedlings of species, such as, *Centaureum erythraea*, which in the absence of infection remained exceedingly small (Table 2) and eventually died. More important, however, was the change in relative abundance of

canopy dominants and subordinate species. In Table 3 it is apparent that the effect of mycorrhizas was to reduce the yield of the canopy dominant *Festuca ovina* and to stimulate strongly the yields of species such as *Scabiosa columbaria*, *Hieracium pilosella* and *Plantago lanceolata*, all of which were found to have heavy VA infection when the root systems of randomly selected individuals were examined at the end of the experiment.

Two sources of evidence suggest that the improved yields of the subordinate species were not simply due to the reduced vigour or increased root/shoot biomass of the infected canopy dominant. First, the subordinate species *Rumex acetosa* which, in its natural habitats<sup>23</sup> and in our experiment contained no VA infection, did not respond significantly to the mycorrhizal treatment (Table 3). The second source of evidence was obtained at the end of the experiment, when entire individual shoots of the canopy dominant *Festuca ovina* in selected mycorrhizal and non-mycorrhizal microcosms were sealed into transparent boxes

**Table 4** Transfer of  $^{14}\text{C}$  label to neighbouring plants in inoculated and uninoculated microcosms

| Species                     | Inoculated (mycorrhizal)  |                     | Uninoculated (non-mycorrhizal) |                     |
|-----------------------------|---------------------------|---------------------|--------------------------------|---------------------|
|                             | % Root length<br>infected | Shoot radioactivity | % Root length<br>infected      | Shoot radioactivity |
| <i>Festuca ovina</i>        | 89                        | 9,276 $\pm$ 3,104   | 0                              | 622 $\pm$ 387       |
| <i>Briza media</i>          | 77                        | 14,002 $\pm$ 860    | 0                              | 1,136 $\pm$ 572     |
| <i>Poa pratensis</i>        | 54                        | 6,241 $\pm$ 2,901   | 0                              | 404 $\pm$ 122       |
| <i>Plantago lanceolata</i>  | 67                        | 18,764 $\pm$ 2,390  | 0                              | 730 $\pm$ 93        |
| <i>Hieracium pilosella</i>  | 59                        | 60,719 $\pm$ 3,326  | 0                              | 297 $\pm$ 101       |
| <i>Centaurea nigra</i>      | 64                        | 45,081 $\pm$ 1,891  | 0                              | 1,338 $\pm$ 140     |
| <i>Leontodon hispidus</i>   | 70                        | 15,363 $\pm$ 3,540  | 0                              | 546 $\pm$ 247       |
| <i>Scabiosa columbaria</i>  | 86                        | 23,912 $\pm$ 5,673  | 0                              | 786 $\pm$ 290       |
| <i>Centaureum erythraea</i> | 44                        | 4,213 $\pm$ 1,754   | —                              | —                   |
| <i>Rumex acetosa</i>        | 0                         | 494 $\pm$ 250       | 0                              | 376 $\pm$ 213       |

Levels of mycorrhizal infection (%) and of shoot radioactivity (d.p.m. per mg dry weight with 95% confidence limits) in plants grown in the inoculated or uninoculated condition in microcosms in which the shoot of a neighbouring individual of *Festuca ovina* was supplied with 3,700 kBq of  $^{14}\text{CO}_2$ . Plants were harvested 72 h after commencement of isotope supply to donor plants, — Insufficient survivors for measurement.

and exposed to 3,700 kBq of  $^{14}\text{CO}_2$ ; after 72 h, radioactivity in surrounding individuals was, in all cases, much higher in infected plants (Table 4). In *Rumex acetosa* which did not become infected in the mycorrhizal microcosms and in all of the plants of the non-mycorrhizal microcosms levels of radioactivity were very low. This shows that  $^{14}\text{CO}_2$  released by leakage or respiration did not contribute significantly to the process of incorporation and supports earlier suggestions<sup>11,19,24-27</sup> that export of assimilate from 'source' (canopy dominants) to 'sink' (understorey components) through a common mycorrhizal network may be an important element of the mechanism maintaining species-rich communities in infertile soils.

The reduced yield of the canopy dominant *Festuca ovina* may be an inevitable consequence of the allocation of carbon to the mycorrhizal mycelial network in the soil. Over the time-span of the experiment infection thus appears to have been a disadvantage to this species; in the long term however the established plant may gain some compensation for infection in the form of enhanced mineral nutrient supply provided by the fungus. It is also possible that under natural conditions a major benefit of infection could arise through seedlings of *Festuca ovina* acting as sinks for assimilate during their establishment; persistence over a long juvenile phase is characteristic of regeneration in canopy dominants of infertile soils<sup>9,28</sup>.

Table 3 reveals that in subordinate species of low stature, such as *Scabiosa columbaria*, *Silene nutans*, the beneficial effects of grazing and mycorrhizal infection upon yield were compounded. However, in the taller subordinates, *Briza media* and *Plantago lanceolata*, the effect of grazing was to nullify the influence of infection. It is also evident that the combined impact of grazing and mycorrhizas upon the yield of the canopy dominant *Festuca ovina* was greater than that of either of the two factors acting in isolation.

The failure of the investigation to provide evidence of spatial niche differentiation runs counter to current equilibrium models<sup>17</sup> of species diversity but should be treated with caution because the results may depend critically upon the type of soil heterogeneity applied, the species included in the study, the relatively constant climatic environment and the limited duration of the experiment.

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## Competition favouring inactive over active motor neurons during synapse elimination

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During normal postnatal maturation, mammalian muscles undergo an orderly process of synapse elimination, whereby each muscle fibre loses all but one of the multiple inputs with which it is endowed at birth<sup>1</sup>. Experimental perturbations that increase or decrease the overall activity of nerve and/or muscle cause a corresponding increase or decrease in the overall rate of neuromuscular synapse elimination<sup>2-7</sup>. On other grounds it has been suggested that competition among motor neurons is important in determining which synapses survive and which are eliminated<sup>1,8-10</sup>. Would a difference in activity among the terminals at the same endplate affect the outcome of the competition and not just its rate? We investigated this issue by blocking activity for four days in a small fraction of the motor neurons innervating the neonatal rabbit soleus muscle. Twitch tensions of motor units were subsequently measured for both the active and inactive populations of neurons to assess whether the inactive neurons had lost fewer or more synapses than is normal. We found that inactive motor neurons have a significant advantage compared to active counterparts in control experiments, a finding opposite to that expected if the neuromuscular junction operated by classical 'Hebbian' rules of competition<sup>11</sup>.

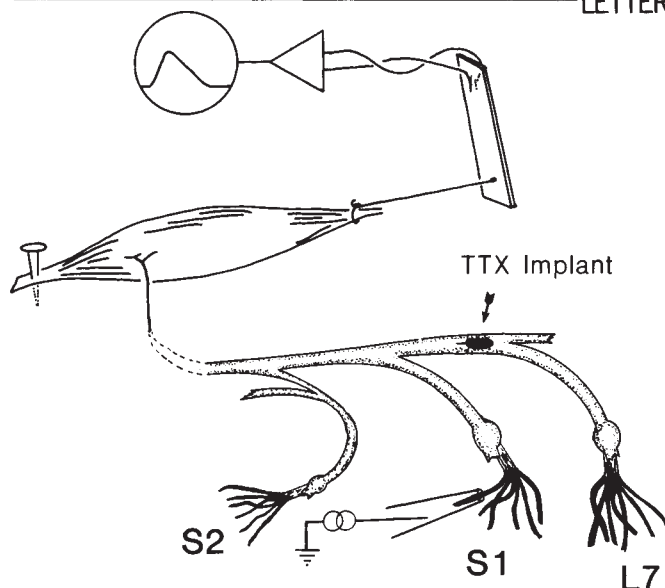
Our experiments were designed to meet three major constraints. First, it was necessary that the differential activity be imposed during a period when synapse elimination occurs rapidly (constraint 1). It was also essential to have two physically separable populations of motor axons whose synapses initially are heavily intermixed, so that active and inactive synapses would be in direct competition at individual endplates (constraint 2). Finally it was important that postsynaptic activity persist in all muscle fibres when the differential nerve block was imposed, so that synaptic competition would not be confounded by the tendency of inactive muscle fibres to induce nerve sprouting<sup>12</sup> (constraint 3).

Our experimental design took advantage of the fact that the rabbit soleus muscle is innervated primarily through the spinal root S1, but with a minority of inputs deriving from one or both of the 'extreme' roots, L7 and S2. In rabbits of postnatal age 4 or 5 days, a Silastic plug laden with tetrodotoxin (TTX) was surgically implanted into spinal nerve L7 or S2 proximal to its junction with S1 (Fig. 1). The TTX was released slowly from the plug, causing a continuous blockade of impulse conduction that was restricted to the implanted nerve. The soleus muscle normally undergoes rapid synapse elimination during this period<sup>13,14</sup>, thereby satisfying constraint 1.

To assess whether the remaining two constraints were met, we measured the proportion of the soleus muscle that was innervated through each of the three ventral roots (L7, S1 or S2) in a population of 12 normal animals aged 4-5 days, the time of the implant. In all nine cases where the extreme root contained 18 or fewer (range 6-18) soleus motor axons, there was nearly complete overlap between the extreme and middle (S1) roots, insofar as the middle root innervated virtually the entire muscle (within the 3% error associated with fluctuations in peak tension measurements). We report here only on cases where the relevant extreme root included fewer than 18 motor axons (range 2-17, mean 6, for 9 TTX-implanted roots). In these cases we can infer that nearly all TTX-inactivated synapses were

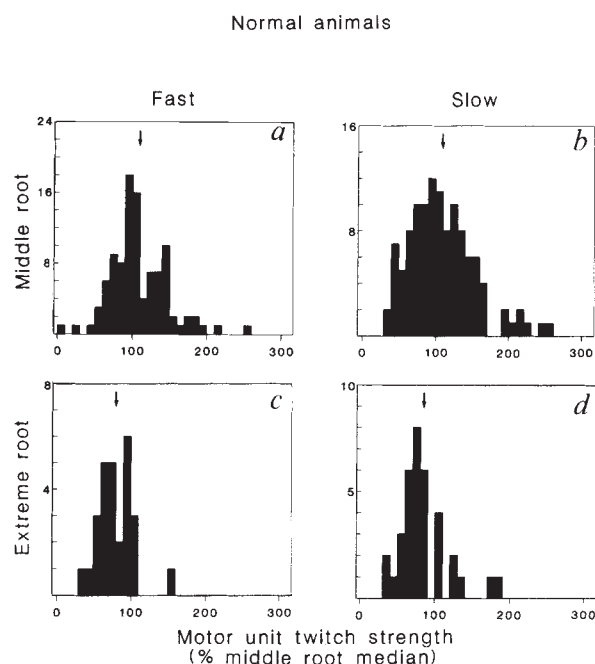
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**Fig. 1** Diagram of the experimental preparation, the rabbit soleus and its innervation through different spinal roots (L7, S1, S2). Conventional symbols show stimulating electrode (bottom), transducer, amplifier and oscilloscope (above). A Silastic plug (control or tetrodotoxin laden, see below) was implanted beneath the epineurium of the L7 or S2 spinal nerve (L7 implant shown) through a small hole made with a fine tungsten needle. In preparations dissected four days after implantation, motor unit twitch tensions were measured *in vitro*, using a force transducer attached to the muscle by a length of 6-0 silk thread and graded stimulation of ventral root filaments by a fine suction electrode. Based on the bimodal distribution of twitch rise times, motor units were segregated into groups. The natural split points varied somewhat among animals, ranging from 257 to 295 ms except for two cases in which the splits were at 230 and 320 ms. Contraction times (mean  $\pm$  s.e.m.) were  $219 \pm 4$  ms for fast motor units and  $363 \pm 6$  ms for slow motor units. These values did not vary significantly across experimental groups. Contraction speeds were much slower than normal *in vivo* speeds owing to the lower temperature ( $19.0 \pm 0.5^\circ\text{C}$ ) for our *in vitro* experiments. Individual animals of the same age vary both in numbers of soleus motor neurons and in degree of polyinnervation. Consequently, average motor unit size (as percentage of maximal whole-muscle tension) spanned a wide range (generally two- to fourfold) for both fast and slow, among different animals of the same age, in all three groups (normal, control and TTX implants, see Fig. 4 legend). We therefore scaled motor unit sizes from each animal to the median fast or slow motor unit size from the middle root (values termed % of median). Scaling was done to median rather than mean because size distributions were slightly skewed from a normal distribution and because statistical analyses were carried out using non-parametric tests.

**Methods.** Tetrodotoxin-laden plugs were made by application of a collagen/ $\alpha$ -cellulose suspension onto the tip of a 2-3-mm length of 40-gauge platinum wire. After about 15 min drying, a 0.20-0.23  $\mu\text{l}$  drop of 10 mM tetrodotoxin was applied to the collagen/ $\alpha$ -cellulose and allowed to dry for 1 h. The plug was then dipped into Silastic (Dow-Corning) thinned with xylene to form a smooth, even coat around the TTX-impregnated matrix. Plugs were cured for 3 days at room temperature and for 4-25 days at  $5^\circ\text{C}$  before implantation. Control plugs were made in an identical manner except that no tetrodotoxin was applied. In addition to the *in vitro* assay for the effectiveness of TTX block, animals were assayed *in vivo* by comparison of the relative resistances to flexion or extension for the left foot (implanted side) with the right foot and by observation of foot position at rest. There was no reliable *in vivo* assay for implants of the S2 spinal nerve, which is small and innervates primarily tail musculature. However, for animals in which other nerves were implanted (L7 and sometimes S1 or L6 inadvertently), *in vivo* assessments were in good agreement with the subsequent *in vitro* observations. Out of 16 animals which were blocked *in vitro*, 14 had shown evidence of block *in vivo* for the full 4 days. Of the other 2 animals, one, a block of L7, was clearly blocked *in vivo* for 3 days; the other was a block of S1 which showed only weak evidence of *in vivo* block for 1-2 days.



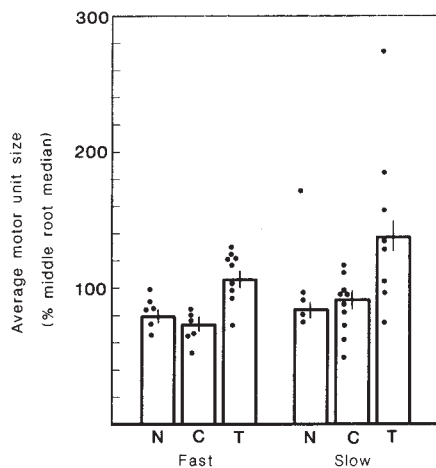
**Fig. 2** Histograms showing distribution of fast (a, c) and slow (b, d) motor unit sizes from middle (a, b) and extreme (c, d) roots of six normal 8- and 9-day-old animals. Fast, middle motor units (a) are significantly larger than fast, extreme motor units (c,  $108 \pm 4$  versus  $79 \pm 5$ ,  $P < 0.001$ , Mann-Whitney *U*-test). Slow, middle motor units (b) are significantly larger than slow, extreme motor units (d,  $109 \pm 5$  versus  $84 \pm 6$ ,  $P < 0.001$ ). There was no significant size difference between extreme-rostral and extreme-caudal motor units for either the fast or slow population (not shown). Arrows, mean motor unit sizes.

in direct competition with the active synapses supplied through the middle root (satisfying constraint 2), and that virtually every muscle fibre remained active because of the extent of innervation by the active root(s) (satisfying constraint 3).

Four days after implantation, the soleus muscle was dissected free along with its innervation back to the ventral roots. The effectiveness of the TTX block was assayed *in vitro* by stimulation of the implanted nerve at points both proximal and distal to the TTX plug. Nine animals in which the TTX blockade was complete were used for further analysis following removal of the plug and washout of TTX (20-150 min) to restore conduction. Ten animals implanted with control plugs (containing no TTX) and six normal 8-9-day-old animals were also analysed. Motor units were separated into fast and slow groups based on twitch rise times<sup>15</sup>. Motor unit sizes were estimated from twitch tensions, expressed as a percentage of the twitch tension elicited by supramaximal, direct electrical stimulation of the whole muscle (percentage of maximum, under 'B' in Table 1)<sup>14,15</sup>. For much of the analysis, motor unit sizes were subsequently normalized to the median fast or slow value from the middle spinal nerve for each animal (% median, under 'A' in Table 1). By compensating for the fact that average motor unit size varies substantially among individuals of the same age (see Fig. 1 and Fig. 4 legends), this normalization step allowed us to pool data from many animals when testing for small differences between extreme and middle roots.

Results from normal animals at 8-9 days of age revealed an unexpected bias in motor unit sizes in that motor units from extreme roots were on average only ~75% as large as those from the middle root (Fig. 2, means indicated by arrows; Table 1, compare row 4A with row 3A). This difference was highly significant for both the fast population (79% against 108%) and the slow population (84% against 109%). No such difference had been noted in a previous report which did not analyse fast and slow motor unit size separately and which compared rostral





**Fig. 3** Motor unit size (as % of middle root median) for normal extreme roots (N), control implanted extreme roots (C) and inactive, TTX implanted extreme roots (T). Bars, mean  $\pm$  s.e.m. range. Left, comparisons for fast populations; right, those for slow. Solid dots, mean sizes of extreme root motor units from individual animals. Control implanted motor units do not vary significantly in mean size from normal motor units (see Table 1 for mean values and statistical significance). The entry at 172% for the slow population of normal animals is from an animal whose extreme root contributed just one slow motor unit. Mean motor unit sizes for control implanted motor units are significantly smaller than inactive, TTX, implanted motor units (see Table 1 for mean values and statistical significance).

to caudal motor units rather than extreme to middle<sup>14</sup>. Further experiments have shown that the difference observed in the present study persists through the completion of synapse elimination (11–15-day-old animals), but that no difference is detectable in heavily polyinnervated (4–5-day-old) animals<sup>16</sup>. Thus, motor neurons with with extreme spinal positions normally lose significantly more synapses than those from middle positions during normal maturation.

Distributions of motor unit sizes for animals implanted with control plugs were similar in all respects to those for normal 8–9-day animals (Fig. 3 and compare Table 1 row 6A with 4A). In particular, implantation of control plugs into extreme roots

had no significant effect on average motor unit size, as indicated in Fig. 3 by the mean motor unit sizes from individual animals (dots) as well as the mean size for all animals pooled in each group (bars). In contrast, implantation of TTX plugs into extreme roots resulted in motor units that on average were larger by about 50% than the corresponding active units in control implanted and normal animals (Fig. 3; Table 1, row 8A versus 6A and 4A). In addition, Fig. 4 shows the data for individual motor units from extreme roots of all control and TTX-implanted animals. Motor units are grouped into vertical columns according to the animal from which they were measured. There is a wide range of motor unit size for both the control and TTX-implanted units, but the distribution is shifted toward greater values for the TTX-implanted groups. The differences of the means were highly statistically significant ( $P < 0.001$ ) for both the fast motor unit population (106% versus 73%) and the slow population (137% versus 91%). Moreover, in eight out of nine cases, the mean size for the TTX-implanted animals was larger than the overall mean for the control groups (Fig. 3). The only exception was a case with only one fast and one slow motor unit from the inactivated root (animal 'n' of Fig. 4); we attribute this instance to statistical fluctuation which is to be expected in view of the wide range of normal motor unit sizes at the time of implantation (four- to five-fold, data not shown).

From comparison with their mean sizes at 4–5 days, the inactive neurons still lost many of their synapses by 8–9 days (27% for fast units, 46% for slow; from Table 1, rows 1B and 8B). The percentage normally lost through the extreme roots during this period is 42% for fast and 59% for slow (Table 1, row 1B versus 4B), implying that the inactive neurons lost significantly fewer synapses than is normal. Because the middle root (active) motor units from TTX-implanted animals were not larger than normal (Table 1, row 7B versus 3B and 5B), we can rule out the possibility that synapse elimination was slowed significantly over the entire muscle by systemic effects of TTX<sup>17</sup> or decreased overall activity.

Although we infer that the differential effect on motor unit sizes is attributable specifically to inactivity in the TTX-implanted root, it does not necessarily follow that this represents an effect on the competitive ability of motor neurons. Alternatively, there might have been a simple slowing of synapse elimination among the subset of muscle fibres innervated by the TTX-implanted root. For example, this could conceivably occur

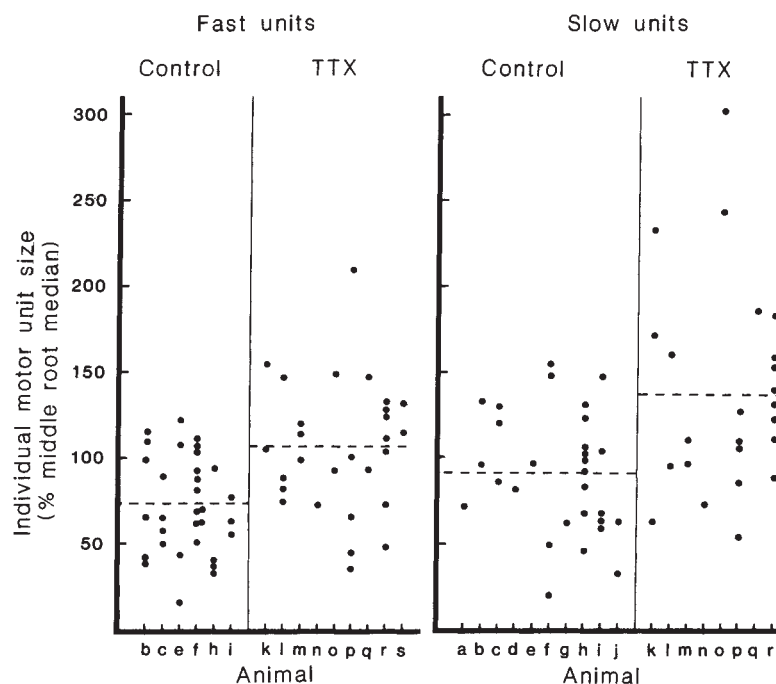
**Table 1** Motor unit sizes by age, root of origin, and experimental condition

|   |                  | Motor unit size     |                      |                      |                      |
|---|------------------|---------------------|----------------------|----------------------|----------------------|
|   |                  | A: as % median      |                      | B: as % maximum      |                      |
|   |                  | Fast                | Slow                 | Fast                 | Slow                 |
| 1 | Normal 4–5-day   |                     |                      |                      |                      |
|   | All roots        | —                   | —                    | 6.7 $\pm$ 0.33 (8)   | 3.9 $\pm$ 0.49 (8)   |
| 2 | Normal 14–15-day |                     |                      |                      |                      |
|   | Middle roots     | —                   | —                    | 3.0 $\pm$ 0.23 (5)   | 0.92 $\pm$ 0.02 (5)  |
| 3 | Normal 8–9-day:  |                     |                      |                      |                      |
|   | Middle roots     | 108 $\pm$ 3.8 (101) | 109 $\pm$ 5.1 (115)  | 5.0 $\pm$ 0.49 (6)   | 1.9 $\pm$ 0.33 (6)   |
| 4 | Extreme roots    | 79 $\pm$ 4.6 (27)*  | 84 $\pm$ 5.6 (35)*   | 3.9 $\pm$ 0.30 (6)   | 1.6 $\pm$ 0.17 (6)   |
| 5 | Control animals: |                     |                      |                      |                      |
|   | Middle roots     | 100 $\pm$ 2.6 (201) | 101 $\pm$ 3.1 (165)  | 5.7 $\pm$ 0.66 (10)§ | 2.3 $\pm$ 0.29 (10)§ |
| 6 | Extreme roots    | 73 $\pm$ 5.1 (32)†  | 91 $\pm$ 6.5 (29)†   | 3.9 $\pm$ 0.47 (10)  | 1.8 $\pm$ 0.21 (10)  |
| 7 | TTX animals:     |                     |                      |                      |                      |
|   | Middle roots     | 102 $\pm$ 3.0 (165) | 105 $\pm$ 4.9 (105)  | 4.6 $\pm$ 0.64 (9)§  | 1.6 $\pm$ 0.21 (9)§  |
| 8 | Extreme roots    | 106 $\pm$ 7.2 (28)‡ | 137 $\pm$ 11.3 (26)‡ | 4.9 $\pm$ 0.81 (9)   | 2.1 $\pm$ 0.33 (9)   |

Values given as '% median' are mean  $\pm$  s.e.m. of the pooled motor unit sizes, expressed as a percentage of the middle root median, from all animals in the group. Numbers in parentheses are number of motor units. Values from middle roots exceed 100% because distributions of motor unit sizes are skewed such that the mean is greater than the median (see Fig. 2). Values given as '% maximum' are mean  $\pm$  s.e.m. of the mean values from each animal in the group. Numbers in parentheses represent the number of animals (means) in each group. \* Smaller than normal, middle root,  $P < 0.001$ . † Equal to normal, extreme root,  $P > 0.2$ . ‡ Larger than control implanted, extreme root,  $P < 0.001$ . § Equal to normal, middle root,  $P > 0.2$ . || Equal to control implanted, middle root,  $P > 0.2$ . ¶ Smaller than control implanted, middle root,  $0.02 < P < 0.05$ . All  $P$  values determined from Mann-Whitney U-test.

**Fig. 4** Comparisons of individual motor unit sizes (% middle root median) from TTX-implanted and control-implanted extreme roots plotted according to the animal from which they were measured. Dashed lines, mean sizes of pooled motor units from each group. Animals ordered according to the average size of fast motor units (percentage maximum) for the middle root, with lowest values on the left. These values range from 3.3 to 9.5% for fast units from control animals, from 2.8 to 8.7% for fast units from TTX-implanted animals; from 1.0 to 3.7% for slow units from control animals, and from 0.9 to 2.7% for slow units from TTX-implanted animals.

**Method.** For animals in which the percentage of the muscle innervated by the middle and extreme roots was measured, we estimated the proportion of the fibers innervated by the extreme root that were not innervated by any other root (usually just the S1 root). This number ( $z$ , the 'per cent exclusive innervation') was calculated from the equation  $z = 100 \times (100 - x) / y$ , where  $x$  is the percentage of muscle fibres innervated by the middle root and  $y$  the percentage innervated by the extreme root. Measurement of  $x$  is subject to variations of  $\pm 3\%$  due to slight fluctuations in the response to direct muscle stimulation. Therefore,  $z$  is most prone to error when the numerator is small ( $x > 95\%$ ), which occurs when  $y$ , the extreme root innervation, is also small. Our exclusion of cases with  $y < 10\%$  (see text) corresponds to extreme roots containing only one or two motor units.



as a consequence of the modest reduction (generally less than 50%) in overall activity among the fibres innervated in part by an inactive motor axon. Slower elimination would lead to motor unit sizes that were slightly elevated on average for the active, middle root and more substantially elevated for the implanted root, relative to control values at this age. In contrast to this prediction, the mean motor unit size (as percentage of maximum) for active (middle root) motor units from TTX-implanted animals was slightly smaller than for corresponding units from normal and control animals (Table 1, row 7B versus 3B and 5B; see also Fig. 4 legend). However, these differences were statistically significant only for the slow motor units when compared to the control animals.

The slowing hypothesis also predicts that polyinnervation would be greater than normal specifically on fibres initially co-innervated through extreme and middle roots. We therefore determined whether the overlap in innervation by extreme and middle roots differed in TTX-implanted and normal animals. For the subset of muscle fibres innervated through the extreme root, we estimated the percentage of them that were innervated exclusively through that root, and were not in addition innervated through the other root(s) ('percent exclusive innervation', Fig. 4 legend). When data from all relevant animals are included in the analysis, the percentage exclusive innervation was  $34 \pm 8\%$  ( $N = 8$ , mean  $\pm$  s.e.m.) for TTX-implanted animals compared to  $19 \pm 8\%$  ( $N = 18$ ) for normal and control animals, corresponding to less overlap in the TTX group. The difference between these mean values is statistically significant ( $P < 0.025$ , Mann-Whitney  $U$ -test). The estimates of percentage exclusive innervation are particularly prone to error when the innervation through the extreme root is small (Fig. 4 legend). When animals in which the extreme root innervated less than 10% of the muscle are excluded, the difference is more significant ( $36 \pm 9\%$ ,  $N = 7$  for TTX-implanted animals compared to  $12 \pm 4\%$ ,  $N = 13$  for normal and control animals;  $P < 0.01$ ). This is the result expected if the inactive neurons outcompete their active rivals and synapse elimination proceeds at a normal rate at the endplates where the competition occurs. We therefore favour the interpretation that inactivity increases the competitive ability of the synapses of a motor neuron relative to those of active motor neurons.

The relatively large value for percentage exclusive innervation in the TTX animals at day 8-9 contrasts with the much lower

value (indistinguishable from zero) in normal 4-5 day animals (implied by the almost complete overlap between middle and extreme roots noted earlier). Evidently a synapse can be eliminated from an endplate (or at least rendered subthreshold) even if it is the last active synapse at that endplate. The failure of active motor axons to reinnervate the resultant inactive muscle fibres may reflect a reduced capacity of neonatal motor neurons to respond to perturbations that induce vigorous sprouting in the adult<sup>4</sup>. One-to-one connectivity also develops (albeit with a substantial delay) in mouse gluteus muscles whose inputs have been completely inactivated by botulinum toxin<sup>18</sup>. Thus, the establishment of single innervation appears to depend on competitive interactions that can proceed between synapses even if some are inactive.

Other investigators have approached the question of differential activity in neuromuscular competition. Ribchester and Taxt applied TTX to one of the two nerves to the rat lumbrical muscle during reinnervation in adult animals<sup>19</sup>. They found that motor units regenerated from the active nerve were more than twice the size of those from the inactive nerve. As they noted, this outcome may reflect differential sprouting of the motor axons in response to cues from inactive muscle fibres. Insofar as our findings result from differences in synapse elimination rather than synapse formation and sprouting, there is no outright conflict between the two studies. In a more direct approach, Ridge and Betz studied synapse elimination in neo-natal rat lumbrical muscles during differential activity produced by electrical stimulation of one of its two nerves<sup>20</sup>. Based on a small sample of stimulated axons, they reported that more active motor units were at a slight advantage. A more convincing case of preferential retention of active versus inactive synapses has been reported for chick ciliary (parasympathetic) neurons innervating skeletal muscle *in vitro*<sup>21</sup>. Thus, the role of activity in neuromuscular competition may depend on the species or type of motor neuron and muscle concerned.

In classical Hebbian synapses, simultaneous pre- and post-synaptic activity is assumed to confer a competitive advantage, manifested by strengthening of more active inputs<sup>11</sup>. Direct evidence for Hebb-like synapses in the central nervous system has been obtained in the hippocampus<sup>22</sup>. Indirect evidence suggestive of Hebbian synapses has been obtained during post-natal maturation in the mammalian visual cortex<sup>23-25</sup>. Our results are the opposite of these and the first indication of a pathway

that endows less active inputs with an outright advantage. In this regard, it is significant that activity rapidly suppresses neurite elongation in cultured invertebrate neurons<sup>26</sup>. An effect of this type would presumably put more active neurons at a disadvantage if it occurs during synaptic competition. A context in which such an anti-Hebbian rule could be functionally useful in the central nervous system has been suggested by Hopfield *et al.*<sup>27</sup>, who showed preferential removal of spurious memory states in simulated neural networks transiently subjected to synaptic weakening during correlated pre- and postsynaptic activity.

In adult mammals there is a correlation between motor unit size and recruitment order<sup>28,29</sup>. This presumably allows for optimal coordination during movement: small motor units are recruited most easily to mediate fine movements, whereas progressively larger motor units are recruited for tasks requiring greater muscular force. If the activity of a motor neuron at birth is related to its adult recruitment threshold, then an advantage for less active neurons during synapse elimination, such as we have demonstrated in these experiments, could help to sculpt the adult configuration from polyinnervated newborn muscles.

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## Protein kinase C injection into hippocampal pyramidal cells elicits features of long term potentiation

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Long-term potentiation (LTP) in the hippocampus is an interesting example of synaptic plasticity because of its induction by physiological discharge rates and its long duration<sup>1,2</sup>. Of the possible biochemical mechanisms that regulate prolonged changes in cell function, protein phosphorylation is a particularly attractive candidate<sup>3</sup>. We have therefore examined the effect of intracellular injection of calcium/diacylglycerol-dependent protein kinase (protein kinase C (PKC)) in CA1 pyramidal neurones in hippocampal slices. Injection of the active enzyme elicited long-lasting enhancement of synaptic transmission, similar to LTP, whereas inactivated kinase failed to do so. The observed changes included an increased amplitude of the excitatory post-synaptic potential (e.p.s.p.) and an increased probability of firing and a reduced latency of the associated action potential.

The effects of long term potentiation and PKC injection on synaptic activation of CA1 cells are compared in Fig. 1. The two types of manipulation caused qualitatively similar effects on the probability and latency of spike discharge. In the experiment shown, injection of PKC not only enhanced the probability of a single action potential, but also recruited a second spike (Fig. 1b), which is not usually seen in LTP.

The temporal development of the PKC-induced changes is shown for another single CA1 cell in Fig. 2. Each point represents the mean of twenty consecutive measurements within a block of 160 seconds. The probability of firing increased from 22 and 18% for the first two time blocks to 100% 18 minutes

after the start of injection (Fig. 2a). The spike latency decreased from a pre-injection level of 9.45 ms to a plateau level of about 6.5 ms. A half-maximal change occurred within 8 minutes (Fig. 2b). The amplitude of the e.p.s.ps, which were normalized to the mean value of the first two blocks, approximately doubled within 25-30 minutes (Fig. 2c). The cell gradually hyperpolarized, first slowly, and reached about 8 mV after 23-33 minutes (Fig. 2d). The input resistance remained unchanged around 26 Mohm for the 20 minutes it could be measured (range 23.4-27.4 Mohm).

Changes similar to those illustrated in Fig. 2 were observed in each of a total of nine cells into which active protein kinase C was injected. Figure 3 presents the average changes in these cells. Each block consists of ten measurements for each cell made within 80 seconds. The average probability of firing of at least one action potential increased greatly, from 44% during the first three time blocks to 92% from 16 to 33 minutes after the injection started (Fig. 3a, filled circles,  $P < 0.01$ ). Remarkably, a majority of the injected cells (8/9) exhibited double spikes and some (3/9) even triple spikes. The average initial spike latency decreased with a high degree of reproducibility (Fig. 3b); a reduction of 1.9 ms was observed between the mean of the first three and of the last five time blocks (two-tailed Student's *t*-test,  $P < 0.001$ ). The mean of the normalized e.p.s.p. amplitudes increased and attained a value of 1.55 between 18 and 27 minutes after start of injection ( $P < 0.001$ ) (Fig. 3c), a finding similar to those seen in LTP experiments. Similar changes in firing probability, spike latency and e.p.s.p. amplitude were seen in response to a separate, but weaker synaptic input.

The soma input resistance was followed from 6 to 20 minutes after the start of injection. The normalized value for all 9 cells remained virtually unchanged (range 92-100% of the 10 minute value). None of the values were significantly different from each other. The change in membrane potential was variable from cell to cell, but the mean value remained unchanged for about 17 min after the start of injection. Afterwards, it showed a gradual hyperpolarization of about 5 mV at around 28 min (Fig. 3d,  $P < 0.001$ ). In contrast, there was a less dramatic effect of enzyme injection on the after-hyperpolarization (AHP), measured 50 ms after a depolarizing pulse (Fig. 3e,  $P < 0.01$ ). AHP measurements after 100 or 800 ms did not show significant changes.

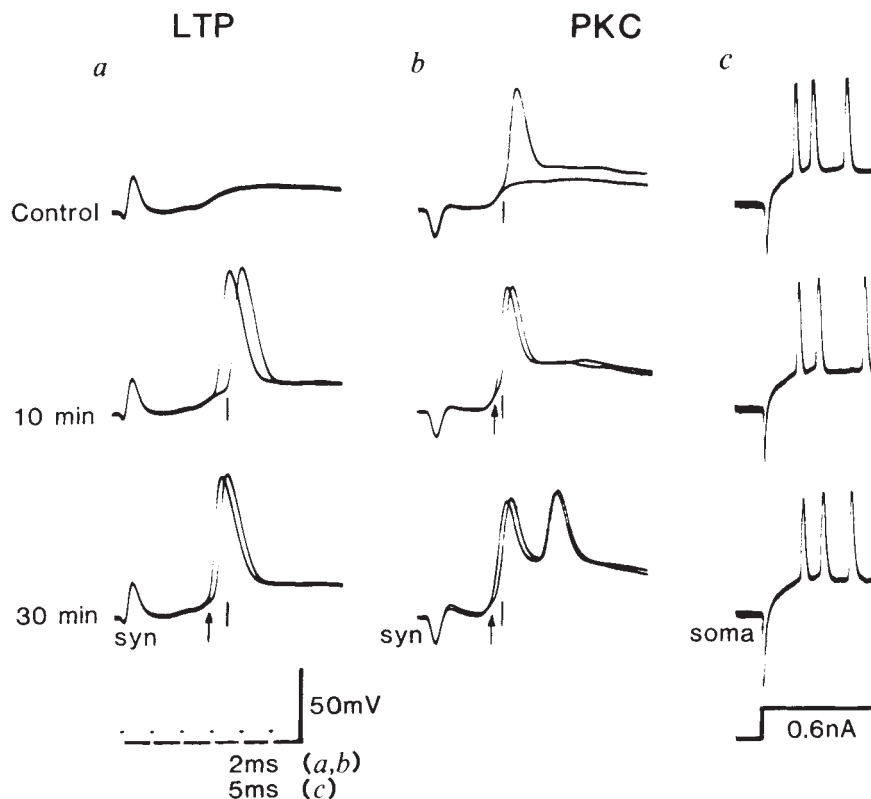
The various effects seen upon injection of active PKC were

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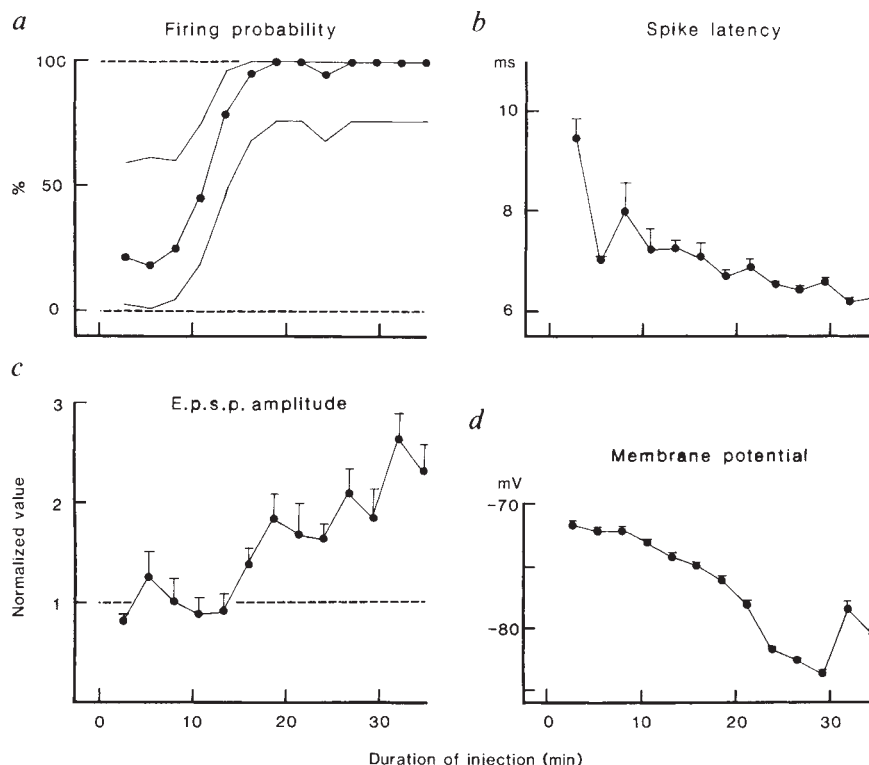


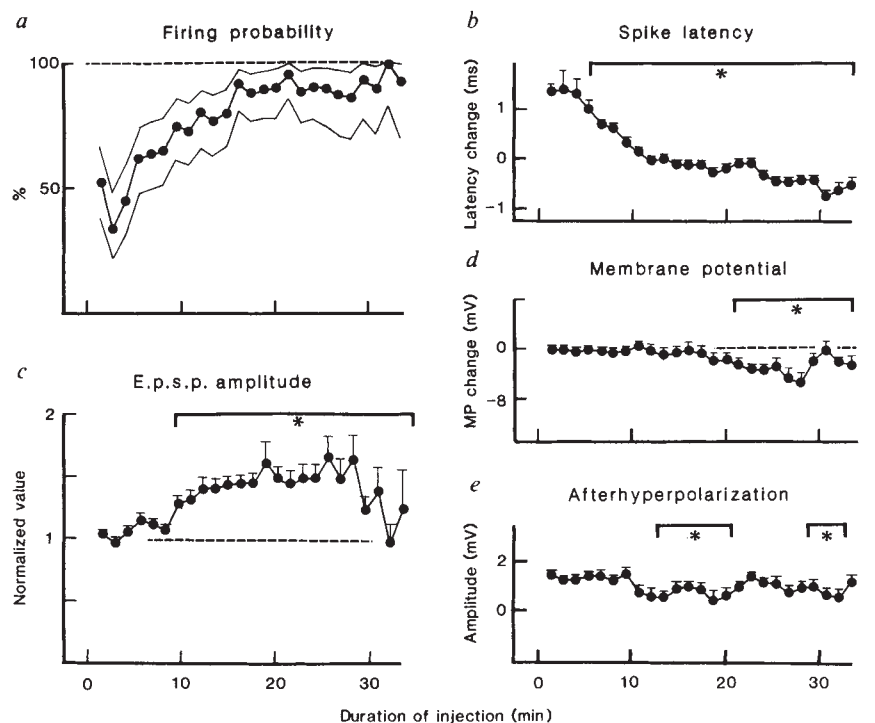
**Fig. 1** Increased synaptic excitability caused by LTP and by injection of (PKC) into hippocampal pyramidal cells. *a*, Intracellular responses from a CA1 pyramidal cell to stimulation of radiatum fibres (200  $\mu$ A, 50  $\mu$ s, 0.125 Hz), before (control), and 10 and 30 min after repeated tetanic stimulation (16 shocks at 100 Hz, 157  $\mu$ A, 50  $\mu$ s, repeated 12 times with intervals of 8 s) show increased probability of discharge and progressive reduction of spike latency (arrow relative to bar). *b*, Enhanced response to synaptic activation (90  $\mu$ A, 50  $\mu$ s, 0.125 Hz) of a CA1 pyramidal cell in another slice, caused by injection of PKC. Upper, middle and lower rows show responses before (control), and 10 (280 nC) and 30 min (840 nC) after the start of injection. *c*, Same cell as *b*. Responses to depolarizing current pulses (0.6 nA, 40 ms, 0.125 Hz) injected across the soma membrane at the same times as in *b*.

**Methods.** Rat transverse hippocampal slices (400  $\mu$ m thick) were placed at the interface between artificial cerebrospinal fluid (ACSF) and humidified gas mixture (5% CO<sub>2</sub>, 95% O<sub>2</sub>)<sup>10</sup>. The ACSF consisted of (in mM): NaCl 124, KCl 2, KH<sub>2</sub>PO<sub>4</sub> 1.25, CaCl<sub>2</sub> 2, MgSO<sub>4</sub> 2, NaHCO<sub>3</sub> 26, glucose 10, bubbled with CO<sub>2</sub>/O<sub>2</sub> gas to keep the pH at 7.4, at 32–34 °C. CA1 pyramidal cells were penetrated with thin-walled microelectrodes filled with a solution of PKC in 0.4 M K-acetate (pH 8.5, 80–180 Mohm) with an activity of 1.3  $\mu$ mol per mg protein per minute. The activity of the protein kinase was measured in solutions containing 1, 0.5, 0.4, 0.3, 0.2 and 0.1 M K-acetate, and showed 20, 52, 68, 77, 85 and 93%, respectively, of the activity in the absence of K-acetate (1.9  $\mu$ mol per mg protein per minute). The enzyme was ejected by negative currents, partly by the holding current and partly by ejection pulses (1–2 nA, 540 ms, 0.125 Hz). For filling electrodes, one vial of enzyme (16  $\mu$ g of PKC per ml) was removed from storage at –70 °C and 50  $\mu$ l was added to 5 ml of 0.4 M K-acetate for back-filling the electrodes by capillary action. The electrodes were kept, tips up, at +4 °C until taken out for use. A sample of the PKC/K-acetate solution was removed for each electrode selected, stored at –70 °C and analysed for enzyme activity compared to the original batch of PKC by the method of Walaas *et al.*<sup>11</sup>, using '87K' protein as substrate<sup>12</sup>. Some of the PKC aliquots were inactivated (to less than 1% of the original activity), by being kept at room temperature for 2 h. Two separate synaptic inputs elicited discharges with a firing probability of around 0.5 and 0.1, respectively. Responses were recorded with an amplifier having a bridge circuit allowing injection of current pulses. The two synaptic inputs and a depolarizing (0.2–1.1 nA, 40 ms) current pulse were delivered alternately at 0.125 Hz. The after-hyperpolarization was measured at 50, 100 and 800 ms after the end of a depolarizing current pulse which produced 2–4 action potentials. All values are expressed as the mean value during either an 80 or a 160 s period.

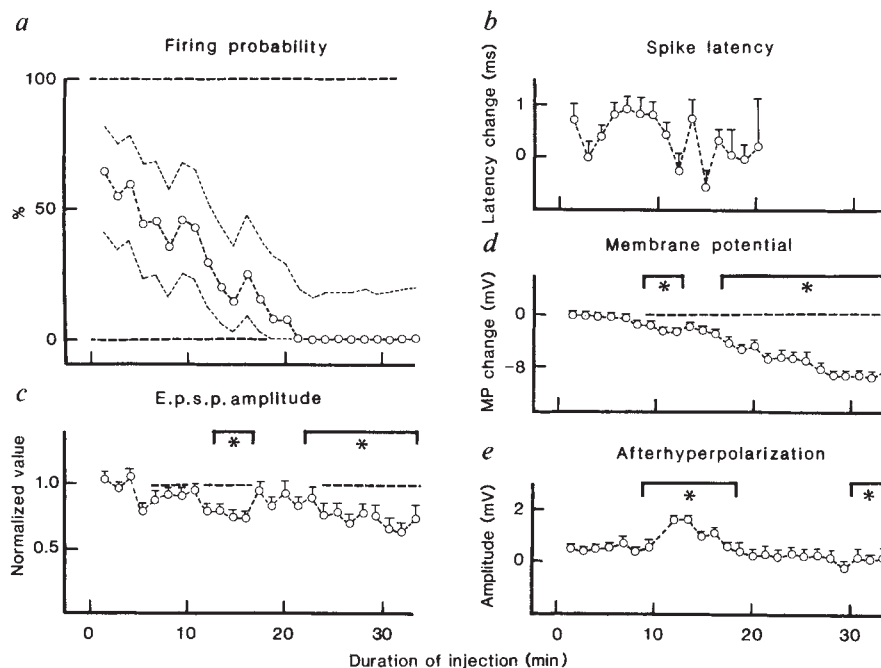


**Fig. 2** PKC-induced changes in a CA1 pyramidal cell. Each filled circle gives the mean value for 20 trials (block of 160 s). The stimulation pulse parameters were 33  $\mu$ A, 50  $\mu$ s and 0.125 Hz. *a*, Firing probability of the initial spike. Thin lines show the 99% confidence intervals based on a binomial distribution. *b*, Action potential latency. The vertical bars give standard error of the mean. *c*, Mean value and standard error of the normalized e.p.s.p.s. *d*, Mean value and standard error of the membrane potential. Criteria for inclusion of cells for analysis were a membrane potential more negative than –60 mV (–72.3  $\pm$  5.8 mV, mean  $\pm$  s.d.) and a spike amplitude above 85 mV (93.9  $\pm$  5.0 mV) which was maintained for at least 15 min after the start of recording. The mean input resistance for cells fulfilling the criteria ( $n = 13$ ) was 30.2  $\pm$  13.9 Mohm. In most cells a steady hyperpolarizing current (0.2–1.0 nA) had to be injected to keep the cell stable throughout the experiment, probably due to the necessity to use low ionic strength electrodes. Because this holding current caused PKC injection, values from the start of the recording period are used as reference for the response changes.





**Fig. 3** PKC-induced changes in cells injected with active enzyme. The filled circles give mean values for nine cells which were injected with enzymatically active PKC. Vertical lines in *b*, *c*, *d* and *e* show the standard error of the mean. Significance is calculated by lumping 3 blocks (each of 80 s) together and comparing them against the first block, using Student's two-tailed *t*-test. An asterisk indicates  $P < 0.001$ . All cells met the criteria described in the legend of Fig. 2. After 17 min there was a gradual decline in the number of cells on which the mean values are based. *a*, Firing probability. The thin lines show the 99% confidence intervals based on a binomial distribution. Second and third spikes are not included. *b*, Changes in the latency to discharge, relative to the mean value for observations made between 15 and 18 min after start. *c*, Changes in normalized e.p.s.p. amplitude. *d*, Resting membrane potential changes. *e*, Afterhyperpolarization amplitude, measured 50 ms after a 40-ms long depolarizing current pulse giving rise to 2–4 action potentials.



**Fig. 4** Cells injected with inactive PKC. Open circles give the mean values for a group of cells ( $n = 4$ ) injected with inactive PKC. *a*–*e*, As Fig. 3, except that the changes in spike latency in *b* are relative to the mean value for observations made between 8 and 12 min after start.

not observed when inactivated PKC was used. For example, four cells injected with inactive kinase showed a gradual reduction of the firing probability and no spike latency change (Fig. 4*a*, *b*). Inactive kinase also failed to show an e.p.s.p. increase; in fact, a small depression was seen (Fig. 4*c*). The activity state of the PKC was not known to the neurophysiological experimenters at the time of injection. Cells injected with inactive kinase showed an even greater hyperpolarization than cells injected with active kinase, possibly ascribable to a better seal around the electrodes. Thus, the membrane potential of the cells which received inactivated protein kinase C turned out to start at a slightly more depolarized level than the cells receiving active enzyme ( $-63.0 \pm 5.2$  mV, mean  $\pm$  s.d., versus  $-69.2 \pm 13.8$  mV). After 25 min the membrane potential of the two groups was similar, measuring  $-73.3 \pm 7.8$  mV (active PKC) and  $-72.6 \pm 4.4$  mV (inactive PKC), respectively. The hyperpolarization of both groups of cells could be attributed wholly or in part to the

necessity of using electrodes which contained a low ionic concentration of K-acetate. Their consequent relative coarseness may explain the slow sealing around the electrodes. The need for a moderate, constant steady negative current injection could also be caused by this fact.

Both pre- and postsynaptic mechanisms contribute to the induction and maintenance of LTP<sup>2,4,5</sup>. Our results show several similarities between the changes seen in LTP and the changes observed following injection of protein kinase C postsynaptically, particularly increased spike probability and reduced latency. In addition, there was an increased e.p.s.p. Some of the e.p.s.p. increase could be due to the observed hyperpolarization. But the e.p.s.p. amplitude increased by 45% during the first 17 minutes without any significant change in the mean membrane potential. The subsequent hyperpolarization of 5 mV would be expected to give an increase of about 0.2 of the normalized e.p.s.p. amplitude based upon measurements from control cells

with comparable input resistance. The observed hyperpolarization can, therefore, only explain a relatively small part of the e.p.s.p. increase. Although the soma input resistance remained unchanged, we cannot rule out an increased dendritic membrane resistance as the cause for the e.p.s.p. increase.

Like the LTP results, the changes induced by PKC injection were only seen when the cells were activated synaptically, leaving the response to depolarizing current pulses to the soma membrane unchanged (Fig. 1c). This observation also suggests a dendritic location for the effect of PKC. Recently, Akers *et al.*<sup>6</sup> found that the total protein kinase C activity was unchanged one hour after LTP induction, but a relative increase of membrane PKC activity, compared to that of the cytosol suggested a translocation of the enzyme. In addition, Malenka *et al.*<sup>7</sup> have reported that addition of 4 $\beta$ -dibutyrate or 4 $\beta$ -diacetate phorbol ester potentiates synaptic transmission, whereas 4 $\beta$ -phorbol and 4 $\alpha$ -phorbol-didecanoate, both inactive phorbol esters, had no such effect. The extracellularly recorded effects were in general similar to those reported here produced by intracellular injection of protein kinase C. Application of phorbol ester has been shown to block a calcium-controlled K<sup>+</sup> current and a Cl<sup>-</sup> current<sup>8,9</sup>. It is expected that PKC-injection would lead to similar changes. The expected increased dendritic membrane resistance could explain part or all of the e.p.s.p. increase observed in the present experiments.

Ideally, one would like to give PKC as a pulse only. The long-lasting effect need not be a durable biological response, but caused by the persistence of the activated enzyme. But at present there is no technique available to stop the PKC activity a certain time after the intracellular injection. In addition, PKC

may have given changes not usually seen in LTP, like the slow hyperpolarization. This discrepancy may partly be explained by the effect of the injected enzyme on central parts of the cell close to the recording electrode, whereas LTP usually is induced in dendritic territories, relatively remote from the soma. Because of cable properties any changes in local membrane potential or resistance appear attenuated as seen by the somatic electrode.

Taken together, these data suggest a role for protein kinase C in LTP. Future experiments should investigate whether other kinases may also be involved, either pre- and/or postsynaptically, in the mechanisms underlying LTP.

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## Host origin of marrow stromal cells following allogeneic bone marrow transplantation

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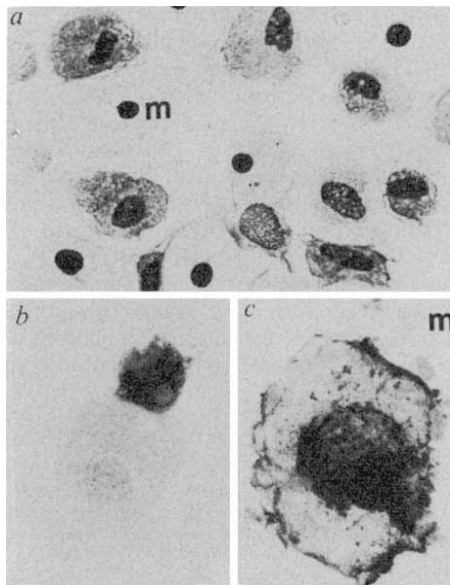
Although it is generally agreed that stromal cells are important in the regulation of haematopoietic cell development<sup>1-3</sup>, the origin of these phenotypically diverse cells has been a subject for debate for more than 50 years<sup>4</sup>. Data which support the concept of a separate origin for the haematopoietic stem cell and the marrow stroma are derived from cytogenetic or enzyme marker studies of explanted and expanded stromal cells grown under conditions that do not allow haematopoiesis *in vitro*<sup>5-7</sup>. Recent evidence in man<sup>8</sup> and in mouse<sup>9</sup> suggesting that the stromal cells capable of transferring the haematopoietic microenvironment *in vitro* are transplantable seemingly questions this dichotomy, one interpretation being the existence of a common haematopoietic/stromal 'stem cell'. We used *in situ* hybridization to discriminate donor cells from host in blood and bone marrow samples obtained from patients with functioning sex-mismatched but HLA-identical allografts. Without exception, marrow-derived stromal cells that proliferate in long-term cultures were found to be of host genotype, whereas the macrophage component of the adherent layer in these cultures originated from the donor.

Heparinized peripheral blood samples and bone marrow aspirates were obtained after informed consent from 10 patients at times ranging from 55 to 1,073 days after marrow transplantation. The clinical information for each patient and details of the transplantation conditioning regimen are outlined in Table 1.

Buffy coat cells from each aspirate were subjected to hypotonic lysis in ammonium chloride and used to establish long-term marrow cultures (see Fig. 1 legend). Cultures were grown at 37°C to accelerate the formation of an adherent stromal cell layer. Under these conditions all bone marrow samples generated within 2-4 weeks a confluent adherent layer consisting of a mixture of fibroblastoid cells, adipocytes and macrophages, as reported by others<sup>8,11</sup>. At confluence the adherent cells were trypsinized and used to make cytocentrifuge preparations for *in situ* hybridization and phenotype analysis.

Wright-Giemsa staining of the trypsinized adherent cell layers consistently demonstrated the predominance of two morphologically distinct cell populations in the long-term cultures derived from marrow transplant patients. One population had the morphology of macrophages with a small heterochromatic nucleus and extensive 'foamy' cytoplasm whereas the remainder were more heterogeneous in appearance, containing larger, more irregularly shaped euchromatic nuclei with two or more prominent nucleoli (Fig. 1a). A clear distinction between these two populations, here termed 'macrophage' and 'stromal' respectively, was made by histochemical (Fig. 1) and immunohistological (Fig. 2) staining of the trypsinized adherent cells from each patient. Macrophages were characterized as strongly butyrate-esterase and acid-phosphatase positive cells expressing the monocyte-macrophage differentiation antigen Leu-M3 (ref. 12) (Fig. 2) in addition to DR as demonstrated by monoclonal antibody P4.1 (ref. 13). Conversely, a high proportion of stromal cells had plasma membrane alkaline phosphatase activity<sup>14</sup> but very weak acid phosphatase and nonspecific esterase activity (Fig. 1b and c) and failed to bind antibody Leu-M3 (Fig. 2). Aside from weak labelling for fibronectin in a small proportion (<5%) of macrophages, the stromal cell population was unique in demonstrating cytoplasmic staining for the extracellular matrix (ECM) proteins laminin, collagen type III, IV and V as shown by immunofluorescence or avidin-biotin immunoperoxidase staining using affinity purified polyclonal sera. Such intracytoplasmic staining is suggestive of synthesis of these various ECM components by the stromal cells, as has



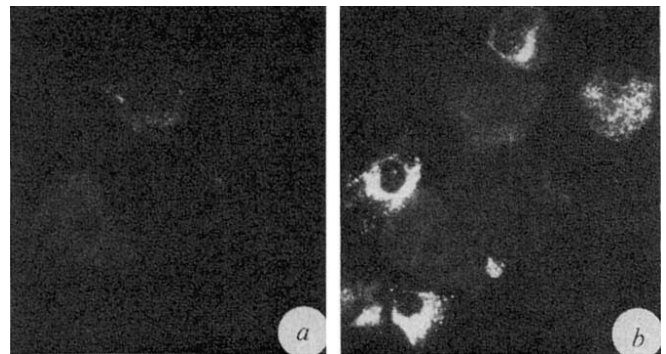


**Fig. 1** Wright-Giemsa stained (a) and nonspecific esterase stained (b) adherent cells from a long-term culture of bone marrow. a, Macrophages (m) are readily distinguished from the more heterogeneous stromal cells by their heterochromatic nuclei and vacuolated cytoplasm. b, A single stromal cell demonstrates only very weak nonspecific (butyrate) esterase activity. c, A large stromal cell exhibits plasma membrane alkaline phosphatase activity; macrophages (m) are negative.

**Methods.** The buffy coat fraction of a bone marrow aspirate was cultured according to a method modified from Gartner and Kaplan<sup>10</sup>. Marrow cells were plated in 25-cm<sup>2</sup> flasks (Corning) at  $2 \times 10^6 \text{ ml}^{-1}$  in 10 ml of complete alpha medium (Gibco) with 12.5% horse serum (Flow), 12.5% fetal calf serum (Hyclone) and 1  $\mu\text{M}$  hydrocortisone sodium succinate. Cultures were incubated at 37 °C in 5% CO<sub>2</sub> and fed weekly by replacement of half of the medium. At 3–4 weeks, the non-adherent cells were washed off with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hank's Balanced Salt Solution. The adherent cells were treated with trypsin-EDTA (Gibco) for 3 min, removed from the flask, washed and then used to make cytospin preparations for Wright-Giemsa and nonspecific esterase stains. Alkaline phosphatase and acid phosphatase activities were demonstrated using standard techniques on preparations fixed with methanol-formaldehyde and citrate-buffered acetone respectively. Magnification  $\times 20$ .

been demonstrated by others<sup>8,15</sup>. Whereas the majority ( $80\% \pm 14$ ) of stromal elements contained interstitial collagen type III suggesting a fibroblast-like phenotype<sup>16</sup>, the additional presence of laminin and collagen type IV in a proportion ( $20\% \pm 5$  and  $55\% \pm 14$  respectively) of stromal cells is consistent with the presence of endothelial cells in the cultures as previously reported<sup>8,11</sup>. This was further confirmed by the presence of collagen type IV (ref. 17) and Factor-VIII-related antigen<sup>18</sup> in a subpopulation of stromal cells demonstrated by two-colour immunofluorescence.

*In situ* hybridization was performed both on the adherent cell cytocentrifuge preparations and on cytospins of blood and bone marrow mononuclear cells obtained at the time of the marrow aspirate using a biotinylated DNA probe specific for repetitive sequences in Y chromatin<sup>19</sup>. Following hybridization the biotinylated probe was detected by indirect immunofluorescence. Cells to which the Y-specific probe hybridized demonstrated a brightly fluorescing spot in the nucleus (Fig. 3). Differential counts for the presence of nuclear fluorescence were performed on at least 500 cells, classified, according to the morphological and immunohistological criteria described above, as macrophages or stromal cells. In all 10 patients receiving marrow transplants from HLA-identical donors of the opposite sex, all



**Fig. 2.** Leu-M3 (a) and intracellular fibronectin (b) in trypsinized adherent layer cells demonstrated using two-colour immunofluorescence. Note the absence of staining for fibronectin in Leu-M3<sup>+</sup> macrophages. Stromal cells containing fibronectin do not express the Leu-M3 antigen.

**Methods.** Cytospin preparations were fixed in acetone, rehydrated in phosphate buffered saline (PBS) and incubated with appropriately diluted phycoerythrin (PE)-conjugated Leu-M3 (Becton Dickinson) and rabbit anti-human cellular fibronectin (from Dr Bill Carter) followed by goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated immunoglobulin G (Tago). Stained preparations were viewed by epifluorescence using filters to visualize fluorescence from PE only (a) or from both fluorochromes (b). Affinity purified goat antibodies against human collagen types III, IV and V (Southern Biotechnology Associates) and rabbit anti-laminin (BRL) were used to visualize these ECM proteins by immunofluorescence. The goat antibodies were detected using swine anti-goat-FITC (Tago) and the preparations counterstained with propidium iodide (Sigma, 5  $\mu\text{g ml}^{-1}$ ) to stain nuclei. Double immunofluorescence staining was performed using rabbit anti-human-Factor-VIII-related antigen (Dakopatts) and goat anti-collagen type IV, followed by swine anti-rabbit-IgG-TRITC (tetramethylrhodamine isothiocyanate) (Dakopatts) and anti-goat IgG-FITC, respectively. Human foreskin fibroblasts and umbilical cord vein endothelial cells were used as negative and positive controls respectively for anti-Factor-VIII-related antigen staining. Magnification,  $\times 20$ .

stromal cells were of host origin, irrespective of the time post-transplant (two months to three years). Conversely, the macrophages associated with the stromal cell layer were found to be exclusively of donor origin, demonstrating the true chimaeric nature of the adherent cells in marrow cultures established from the transplant patients. In all cases examined, the genotype of stromal cells grown in culture was independent of the degree of mixed chimaerism encountered amongst mononuclear cells from the bone marrow and peripheral blood (Table 1).

Our data indicating the failure to transplant detectable numbers of stromal cells in patients receiving bone marrow from siblings of the opposite sex are at variance with those of Keating *et al.*<sup>8</sup> who described a progressive disappearance of host stromal elements with time after transplantation. Several possibilities must be considered for this difference between the two studies. (1) Conditioning of patients before marrow transplantation: although the response of marrow stromal cells to chemotherapy and irradiation is not precisely understood, this seems an unlikely cause because similar regimens of cyclophosphamide and <sup>60</sup>Co irradiation were used in both studies. (2) Criteria for identification of stromal cells: in the study of Keating *et al.*<sup>8</sup>, the composition of stromal cells in the adherent layer was inferred from biochemical analysis of the collagenous proteins synthesized. Analysis of phenotype by histochemical and immunohistological staining as in the current study has the advantage that phenotype and genotype can be accurately correlated at the single-cell level. (3) Method used to determine origin of stromal cells: the reliability of assigning cell origin in



**Fig. 3** *In situ* hybridization for the Y chromosome in adherent cells from a long-term culture of bone marrow from a female patient who received a transplant from a male donor.

**Methods.** A cytospin preparation of adherent cells was fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 10 min and postfixed in 4% paraformaldehyde for 5 min. The slide was overlaid with hybridization mixture [ $2 \times$  SSC (300 mM NaCl, 30 mM sodium citrate), 10% dextran sulphate, 50% formamide,  $0.5 \text{ mg ml}^{-1}$  denatured herring sperm DNA and  $0.75 \text{ } \mu\text{g ml}^{-1}$  plasmid pY3.4 (ref. 19) that was labelled by random primer extension<sup>29</sup> using biotinyl-*N*-aminocaproyl-*N*-5-allyl-dUTP (BRL)] covered with a coverslip and sealed with rubber cement. The cell DNA and probe were denatured *in situ* at  $95^\circ\text{C}$  for 10 min and hybridized at  $42^\circ\text{C}$  for 16 h. The slide was then washed in  $2 \times$  SSC for 10 min at room temperature,  $0.5 \times$  SSC, 50% formamide for 15 min at  $42^\circ\text{C}$ ,  $2 \times$  SSC for 5 min at room temperature twice and in 0.1% HCl in absolute methanol for 5 min. The probe was detected by indirect immunofluorescence with a modification of a method described previously<sup>30</sup>, using an avidin primary, biotinylated anti-avidin secondary and FITC-avidin tertiary and counterstained with Evan's Blue. Magnification,  $\times 20$ .

chimaeras by quinacrine staining is limited by the sensitivity of the technique, especially in situations involving a low degree of mosaicism. Keating *et al.* reported a sensitivity of only 66%. In contrast, *in situ* hybridization has virtually 100% sensitivity and specificity in our hands. (4) Differential detachment of adherent layer cell types with trypsin: when harvesting adherent cells to make cytocentrifuge preparations it was observed that brief exposures to trypsin preferentially released large numbers of macrophages and relatively few stromal cells, the latter requiring longer digestion with trypsin to be detached. Clearly, had care not been taken to ensure the complete removal of all adherent

cells, a sampling bias may have resulted from the differential detachment of macrophages and stromal cells.

The transplantability of bone marrow stromal cells has remained a controversial issue<sup>20</sup>. Bone-marrow derived fibroblast colony-forming cells (CFU-F) have been considered as not transplantable by the intravenous route in mice and man<sup>7,21-23</sup>, although successful lodgement of donor CFU-F in mice has been demonstrated in one instance<sup>24</sup>. CFU-F, however, are only one component of the marrow stroma and may not be fully representative of the phenotypically diverse cell populations that make up the haematopoietic microenvironment *in vivo*. Studies using long-term culture (LTC) established from the marrow of transplanted mice in which an intact haematopoietic microenvironment is believed to develop have documented the failure to engraft donor stromal elements in two instances<sup>25,26</sup> and apparently successful engraftment in another<sup>9</sup>. The latter study should however be interpreted with caution because  $\sim 75\%$  of cells forming the adherent layer in murine LTC are macrophages. A significant proportion of these macrophages cannot be removed by techniques relying on phagocytosis<sup>27</sup>, and could therefore account for the high proportion of donor 'stromal' cells reported.

In conclusion, we have demonstrated the persistence of host stromal cells in patients with sustained marrow grafts from HLA-matched siblings of the opposite sex, indicating that the conditioning regimens used for marrow transplantation do not destroy host stroma. Our inability to detect donor stromal cells could mean either that host stroma must be destroyed before donor stroma can engraft or that the various stromal cell types that proliferate in LTC are not transplantable. Alternatively, it is possible that certain stromal elements in the graft do successfully lodge in the recipient's marrow (albeit at very low frequency) but are not capable of proliferation *in vitro*. An analysis of the bone marrow *in situ* would be required to address this problem. Nevertheless, these data indicate that appropriate regulatory interactions between allogeneic stem cells and stromal cells do occur when the donor and recipient are HLA-identical. It is important now to determine whether the higher incidence of graft failure or rejection seen in HLA-nonidentical or unrelated phenotypically-matched transplants is due to a specific genetic disparity that either prevents requisite interactions between host and donor cells or triggers alloreactivities that destroy one or the other component. Studies designed to address this question are currently in progress.

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**Table 1** Chimaeric status of peripheral blood and bone marrow cells after transplantation

| Patient | Diagnosis     | Days after transplantation | PBMC | Host cells (%) among |                       |                      |
|---------|---------------|----------------------------|------|----------------------|-----------------------|----------------------|
|         |               |                            |      | BMC                  | Adherent stroma cells | Adherent macrophages |
| 3603    | CML           | 55                         | ND   | ND                   | 100                   | 0                    |
| 3545    | Neuroblastoma | 57                         | 0    | 0                    | 100                   | 0                    |
| 3474    | ALL           | 76                         | 0.2  | 6.0                  | 100                   | 0                    |
| 3544    | CML           | 77                         | 0    | 0                    | 100                   | 0                    |
| 3569    | ALL           | 86                         | 0.3  | ND                   | 100                   | 0                    |
| 3445    | Preleukaemia  | 91                         | 2.5  | 8.3                  | 100                   | 0                    |
| 3139    | ALL           | 326                        | ND   | ND                   | 100                   | 0                    |
| 2553    | ANL           | 906                        | 0    | 0                    | 100                   | 0                    |
| 2492    | ANL           | 907                        | 0    | 0                    | 100                   | 0                    |
| 2317    | ANL           | 1,074                      | 0.2  | 2.0                  | 100                   | 0                    |

Abbreviations: PBMC, Ficoll-Hypaque separated peripheral blood mononuclear cells; BMC, bone marrow buffy coat cells; CML, chronic myelogenous leukaemia; ALL, acute lymphocytic leukemia; ANL, acute non-lymphocytic leukemia; ND, not done. Before transplantation, each patient received cyclophosphamide ( $60 \text{ mg per kg}$  for 2 consecutive days) and total body irradiation from opposing  $^{60}\text{Co}$  sources ( $200 \text{ cGy}$  daily for six days,  $200 \text{ cGy}$  twice a day for four days, or  $225 \text{ cGy}$  daily for seven days). Patient 3545 also received one dose of melphalan ( $180 \text{ mg m}^{-2}$  per square metre ( $\text{m}^{-2}$ )). Post-transplant care was as previously described<sup>28</sup>. Adherent cells were prepared as in Fig. 1. A mean of 509 cells per sample were evaluated by *in situ* hybridization as in Fig. 2.



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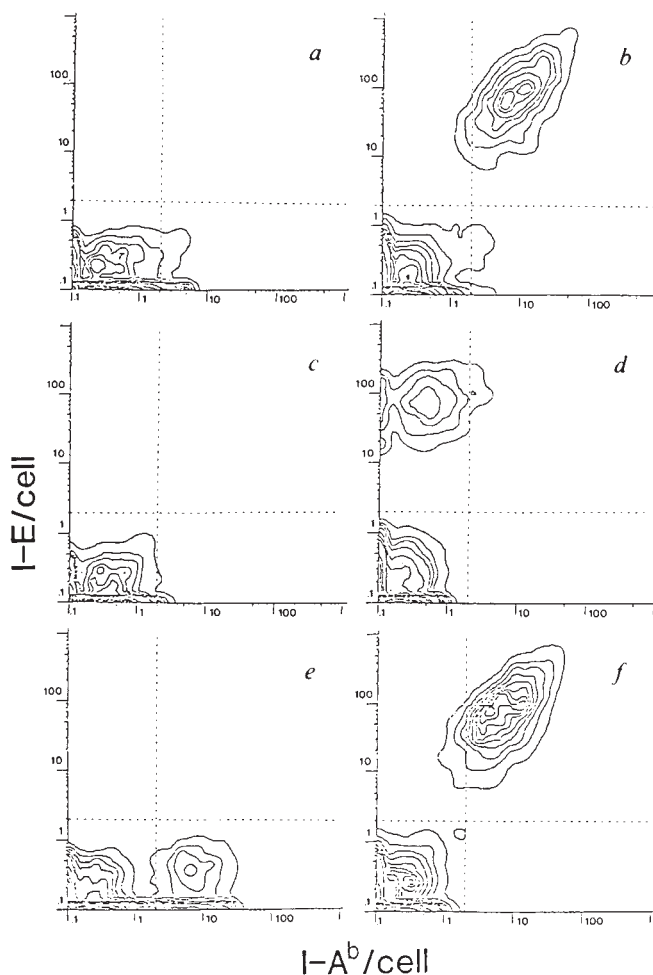
## Prevention of autoimmune insulinitis by expression of I-E molecules in NOD mice

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The NOD (non-obese diabetic) mouse spontaneously develops insulin-dependent diabetes mellitus (IDDM) characterized by autoimmune insulinitis, involving lymphocytic infiltration around and into the islets followed by pancreatic beta ( $\beta$ ) cell destruction<sup>1-5</sup>, similar to human IDDM<sup>6,7</sup>. Genetic analysis in breeding studies between NOD and C57BL/6 mice has demonstrated that two recessive genes on independent chromosomes contribute to the development of insulinitis<sup>8</sup>. One of the two recessive diabetogenic genes was found to be linked to the major histocompatibility complex (MHC)<sup>9</sup>. This is of interest, because the NOD strain has a unique class II MHC: it does not express I-E molecules as no messenger RNA for the  $\alpha$ -chain of I-E is visible in Northern blot analysis; I-A molecules are not detected with any available monoclonal antibodies or by allo-reactive or autoreactive T-cell clones, although their expression is demonstrated with a conventional antiserum to Ia antigens<sup>9</sup>. To examine whether the unusual expression of class II MHC molecules may be responsible for the development of autoimmune insulinitis, we attempted to express I-E molecules in NOD mice selectively, without introducing other genes on chromosome 17 by using I-E-expressing C57BL/6 (B6(Ea<sup>d</sup>)) transgenic mice<sup>10</sup>. We report here that the expression of I-E molecules in NOD mice can prevent the development of autoimmune insulinitis.



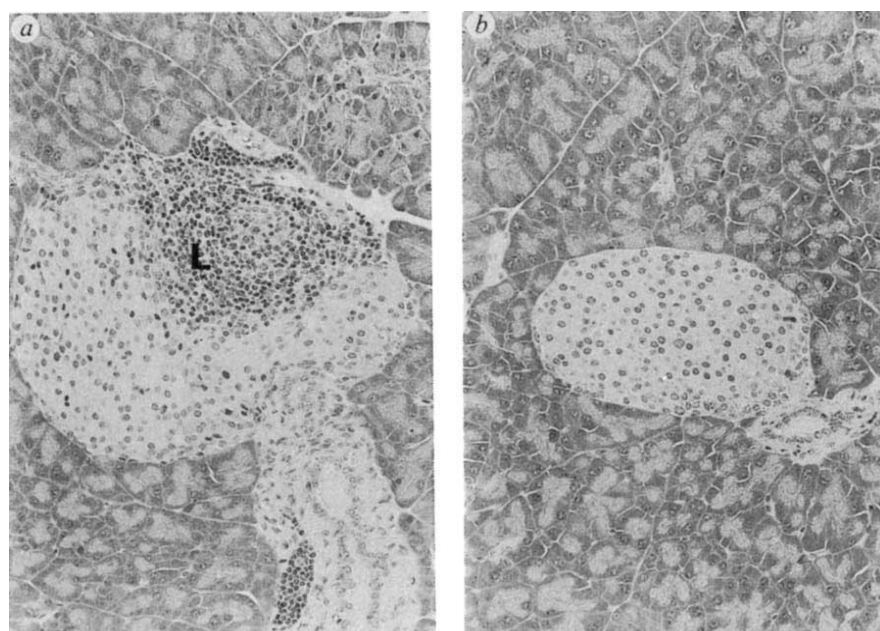
**Fig. 1** The expression of I-A<sup>b</sup> and I-E antigens in NOD, NOD × B6(Ea<sup>d</sup>)F<sub>1</sub> and F<sub>1</sub> × NOD backcross progeny. *a*, NOD mouse; *b*, I-E-positive NOD × B6(Ea<sup>d</sup>)F<sub>1</sub> mouse; *c*, group 1 backcross progeny (lacking both I-A<sup>b</sup> and I-E); *d*, group 2 backcross progeny (lacking I-A<sup>b</sup> but expressing I-E); *e*, group 3 backcross progeny (expressing I-A<sup>b</sup> but not I-E); *f*, group 4 backcross progeny (expressing both I-A<sup>b</sup> and I-E). NOD mice were maintained in the animal colony of Aburaki Laboratory, Shionogi Co. Ltd and mated with B6(Ea<sup>d</sup>) transgenic mice which carry transgenic Ea<sup>d</sup> genes heterozygously. I-E-positive mice were selected by staining peripheral blood lymphocytes (PBL) with an anti-I-E antibody (13/4). I-E-positive F<sub>1</sub> mice were backcrossed to NOD mice. PBL were prepared from mice by a Ficoll-Hypaque gradient centrifugation. Cells were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-I-A<sup>b</sup> monoclonal antibody (28-16-8) and a biotinylated anti-I-E antibody (13/4), developed with Texas-Red avidin and analysed by a dual laser FACS 440 (Becton Dickinson) equipped with an argon ion laser and a dye laser. The x and y axes represent log intensities of green and red fluorescences, respectively.

NOD mice were mated with B6(Ea<sup>d</sup>) mice to obtain F<sub>1</sub> progeny. B6(Ea<sup>d</sup>) mice were prepared by the microinjection of the 14-kilobase (kb) *SacII/XhoI* fragment containing the entire Ea<sup>d</sup> gene sequence into fertilized eggs of B6 mice<sup>10</sup>. It has been shown previously that functional I-E<sup>d/b</sup> (Ea<sup>d</sup>E<sup>b</sup>) antigens are expressed in these transgenic mice<sup>10</sup>. The parental B6(Ea<sup>d</sup>) mice are heterozygous for the Ea<sup>d</sup> gene, and so the F<sub>1</sub> progeny were screened to identify offspring expressing both I-A<sup>b</sup> and I-E molecules. Using the monoclonal antibodies 28-16-8 (anti I-A<sup>b</sup>) and 13/4 (anti I-E<sup>d</sup>), we identified NOD × B6(Ea<sup>d</sup>) F<sub>1</sub> progeny which expressed both I-A<sup>b</sup> and I-E on peripheral blood lymphocytes (PBL; Fig. 1*b*). PBL from parental NOD mice were not stained by either antibody, as expected (Fig. 1*a*). NOD ×

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**Fig. 2** Histopathology of pancreatic islet in the backcross progeny. Staining method was the same as described in the legend to Table 1. *a*, Pancreas from a progeny of group 1 (bearing neither I-A<sup>b</sup> nor I-E). Islet displaying massive lymphocytic infiltration (L). *b*, Pancreas from a group 2 mouse (bearing I-E but not I-A<sup>b</sup>). No infiltration of cells into or around islet is observed.



B6(E $\alpha^d$ ) F1 progeny expressing I-E were backcrossed with NOD mice and 73 female progeny were examined for the expression of I-A<sup>b</sup> and/or I-E molecules and were classified into four groups based on their class II MHC expression. As shown in Fig. 1*c-f*, 19 mice expressed neither I-A<sup>b</sup> nor I-E on their PBL (Group 1, Fig. 1*c*), 18 mice did not express I-A<sup>b</sup> but expressed I-E (Group 2, Fig. 1*d*), 21 mice expressed I-A<sup>b</sup> but not I-E (Group 3, Fig. 1*e*) and 15 mice in Group 4 expressed both I-A<sup>b</sup> and I-E (Group 4, Fig. 1*f*). Of the 37 mice in which I-A<sup>b</sup> was not expressed (Group 1 and 2) 18 expressed I-E instead and 21 of the 36 mice in which I-A<sup>b</sup> was expressed (Group 3 and 4) lacked I-E expression, indicating that the genes for I-A<sup>b</sup> and I-E segregated independently in the backcross progeny. In other words, in B6(E $\alpha^d$ ) transgenic mice the introduced E $\alpha^d$  gene was not integrated into chromosome 17, where the I-A<sup>b</sup> gene is located.

As female NOD mice show accelerated development of insulinitis<sup>8</sup>, only female backcross progeny were examined at 10 weeks of age. As summarized in Table 1, in Group 1 mice, expressing neither I-A<sup>b</sup> nor I-E 9 of the 19 mice (47%) developed insulinitis. Of the mice that express I-E, Group 2 (lacking I-A<sup>b</sup>) and Group 4 (expressing I-A<sup>b</sup>), none of the mice examined (18 mice and 15 mice, respectively) developed insulinitis. In Group 3 (expressing I-A<sup>b</sup> but not I-E) only one of the 21 mice (5%) developed insulinitis. The incidence of insulinitis in NOD  $\times$  B6(E $\alpha^d$ ) F<sub>1</sub> female controls and in parental NOD female mice was 0% and 93%, respectively. The histological features of insulinitis in mice expressing neither I-A<sup>b</sup> nor I-E and islets of non-affected pancreas of mice bearing I-E but not I-A<sup>b</sup> are shown in Fig. 2. The results clearly show that the backcross animals expressing I-E molecules did not develop insulinitis even at 10 weeks of age. About 80% of control NOD mice develop insulinitis at 6 weeks of age<sup>8</sup>. Moreover, no insulinitis was observed in eight of the I-E-bearing mice that lack I-A<sup>b</sup> tested at 25 weeks of age. Thus, I-E expression appears to prevent the onset of insulinitis rather than to delay it. As the E $\alpha^d$  gene was not integrated into chromosome 17, the progeny that lack the I-A<sup>b</sup> antigen were homozygous for chromosome 17 of NOD origin. Therefore, the results indicate that the intact gene for E $\alpha$ , but not other genes on chromosome 17, is required for the prevention of the development of insulinitis.

It has been shown that two recessive genes are involved in the development of insulinitis; one is an MHC-linked gene on

chromosome 17 and the chromosomal localization of the other gene has not been identified<sup>9</sup>. If the E $\alpha^d$  gene in the transgenic mice is integrated on the same chromosome as the second non-MHC recessive gene, then the development of insulinitis in I-E (+) backcross progeny could also be prevented. If this indeed were the case, almost 100% of the backcross mice in Group 1 (lacking both I-A<sup>b</sup> and I-E) should have developed insulinitis, because they should have had the two recessive genes. But as shown in Table 1, only 47% of Group 1 animals developed insulinitis, clearly demonstrating that E $\alpha^d$  and the non-MHC recessive genes are not located on the same chromosome and that the prevention of insulinitis was due to expression of I-E. I-A<sup>b</sup>-bearing mice in the backcross progeny also did not develop insulinitis (Table 1, Group 3). But the prevention by the expression of I-A was not complete as observed in the case of I-E-bearing mice; in addition to the development of insulinitis in 5% of the mice carrying I-A<sup>b</sup> but not I-E, two other mice showed lymphocytic infiltration in the lumen of small lymphatics or periductal connective tissues proximal to the islets (data not shown). Such changes are often observed at the early stages of insulinitis in NOD mice<sup>11</sup>. The effect of I-A<sup>b</sup> expression on the prevention of insulinitis may be essentially the same as that of I-E expression, although it was not complete. In the case of I-A<sup>b</sup> expression,

**Table 1** Incidence of insulinitis in the pancreatic islets of backcross progeny with different phenotypes

|                                   | <i>n</i> | Class II phenotype           | Insulinitis |
|-----------------------------------|----------|------------------------------|-------------|
| BC Group 1                        | 19       | I-A <sup>b</sup> (-), I-E(-) | 9/19 (47%)  |
| 2                                 | 18       | I-A <sup>b</sup> (-), I-E(+) | 0/18 (0%)   |
| 3                                 | 21       | I-A <sup>b</sup> (+), I-E(-) | 1/21 (5%)   |
| 4                                 | 15       | I-A <sup>b</sup> (+), I-E(+) | 0/15 (0%)   |
| F1 NOD $\times$ B6(E $\alpha^d$ ) | 8        | I-A <sup>b</sup> (+), I-E(+) | 0/8 (0%)    |
| Parent NOD                        | 14       | I-A <sup>b</sup> (-), I-E(-) | 13/14 (93%) |

The female mice were killed at 10 weeks of age. The pancreas was removed and fixed in 10% buffered formalin containing 75 mM phosphate buffer, pH 7.0, then embedded in paraffin blocks. Sections (3  $\mu$ m thick) were cut and stained with hematoxylin and eosin. More than 20 islets were examined for each pancreas. A pancreas with lymphocytic infiltration around or into at least more than a single islet was regarded as displaying 'insulinitis'<sup>11</sup>. Each section was analysed by two independent examiners without knowledge of the MHC phenotypes of the mice.

however, chromosome 17 from B6 mice is introduced into the progeny. Therefore, the involvement of other genes on chromosome 17 cannot be theoretically excluded.

The discrimination between self and non-self and the maintenance of the state of tolerance to self antigens are not fully understood. Several studies<sup>12,13</sup> suggest that the MHC class II molecules expressed on thymic epithelial cells or on hematopoietic cells may be responsible for the positive and negative selection of T cells; the positive selection of H-2 restricted T-cell recognition and the negative selection of autoantigen-reactive cells. Here we have directly shown that the expression of I-E molecules can prevent the development of insulinitis. Massive infiltration of CD4-bearing (L3T4<sup>+</sup>) T cells into the pancreas is a characteristic feature in NOD mice (M. Harada, personal communication) and cell transfer experiments have indicated that auto- $\beta$ -cell-reactive helper cells or cytotoxic T cells were responsible for the development of insulinitis in this strain of mice<sup>14</sup>. We have shown that class II MHC molecules are involved in the deletion, inactivation or functional suppression of autoreactive T cells. The mechanism leading to the prevention of insulinitis in I-E-bearing NOD mice is unknown, but possible mechanisms include; (1) I-E molecules may cross-react with an epitope formed by interaction of the  $\beta$ -cell antigen and MHC molecules. Thus, the expression of I-E in the early neonatal stage can make T cells tolerant to autologous  $\beta$ -cells. (2) The expression of I-E induces suppressor cells which prevent the generation of auto- $\beta$ -cell-reactive helper cells. Several experiments<sup>15,16</sup> suggest that I-E molecules are important in the generation of suppressor cells. Future studies with cell transfer experiments will provide information as to the exact mechanism in tolerance induction to autologous  $\beta$  cells by the I-E expression. We have clearly shown that one MHC-linked recessive gene involved in the pathogenesis of insulinitis is the E $\alpha$  gene. Further genetic analyses will be necessary to determine what effect, if any, other MHC-linked genes including I-A have on insulinitis in NOD mice. The BB rat is another experimental model for IDDM and susceptibility to IDDM has also been mapped to the MHC in this strain<sup>17</sup>. Human autoimmune IDDM has also been shown to be strongly associated with HLA-DR 3 or 4 haplotype, particularly in association with DQ or DX region polymorphism<sup>18-20</sup>. These results suggest the involvement of a similar mechanism in human and rat IDDM as well.

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## A cDNA clone from the Duchenne/Becker muscular dystrophy gene

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Duchenne muscular dystrophy (DMD) is the most common of the muscular dystrophies affecting one in 3,000 live male births (see refs 1, 2 for review). Both DMD and the mild form, Becker muscular dystrophy (BMD), are X-linked. There are a number of females affected by the disease who all possess an X-autosome translocation, with the exchange point in the X always occurring within chromosome band Xp21 (refs 3, 4). This, together with linkage and deletion data, has localized the gene at band Xp21 (refs 5-7). DNA fragments from this region have been cloned using a patient with a large Xp21 deletion<sup>8</sup> and from a patient with a t(X;21) translocation<sup>9</sup>. The former clones (pERT 87) comprise the DXS164 locus and the latter clones (XJ) the DXS206 locus. Subclones from both regions allow the detection of deletions in ~11% of DMD patients<sup>8-12</sup>. A fetal muscle complementary DNA clone corresponding to exons in the DXS164 locus has been isolated and detects a 16-kilobase (kb) transcript<sup>13</sup>. We present the isolation of an adult muscle cDNA clone from the DXS206 locus that detects a 16-kb mRNA in adult human muscle. The cDNA clone contains exons that map in the DXS206 locus, the DXS164 locus, and on the centromeric side of these cloned regions. The t(X;21) translocation exchange points occurs within a large intron of 105 kb or larger, indicating that the translocation has disrupted the DMD/BMD gene to cause the disease in this patient.

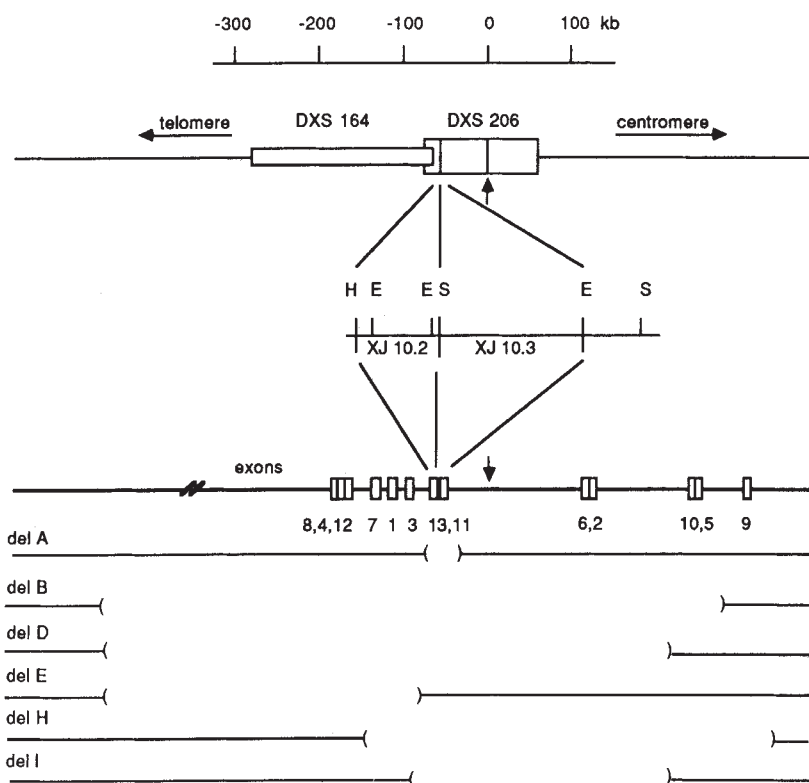
Our original t(X;21) translocation junction clone, XJ1, has been expanded by chromosomal walking to include 120 kb of the surrounding X-chromosomal DNA<sup>14</sup>. This has resulted in joining of the DXS206 locus to the DXS164 locus, yielding a 350-kb contiguous stretch of cloned genomic DNA<sup>14</sup>, as diagrammed in Fig. 1. Several unique sequence fragments from the DXS206 walk clones were isolated and used as probes on cDNA libraries to identify transcribed regions from this locus. Two of these subcloned fragments XJ10.2 and XJ10.3 from walk clone XJ10 (Fig. 1) identified 7 positive clones in 10<sup>6</sup> plaques of an adult muscle  $\lambda$ gt11 cDNA library<sup>15</sup>, kindly provided by Dr Y. Edwards. The largest X-specific cDNA clone was 2.0-kb in size and was shown by subsequent mapping and sequence analysis to contain sequences from XJ10.2 and XJ10.3.

To determine the number of exons represented in the 2.0-kb cDNA clone, a Southern blot of *Eco*RI-digested DNA samples of male, female and a four-X cell line was probed with the 2.0-kb cDNA. There were thirteen hybridizing bands and each gave a hybridization signal proportional to the number of X chromosomes, confirming that the cDNA was X-specific (Fig. 2a). As there are no *Eco*RI sites within the cDNA clone, each genomic *Eco*RI fragment must contain one or more exons of the gene. The *Eco*RI fragments have been numbered E1 to E13. The largest band detected with the cDNA is a doublet which can be resolved on longer gels. Band E11 (1.9 kb) and E13 (0.7 kb) correspond to the *Eco*RI fragments within the XJ10 region (see Fig. 1).

To map the *Eco*RI bands (exons) relative to the t(X;21) translocation junction (arrow in Fig. 1), the 2.0-kb cDNA was hybridized to a Southern blot of DNA from three different somatic cell hybrid lines, one containing the translocated X[der(X)], one containing the translocated 21 [der(21)], both derived from the t(X;21) translocation, and the third containing an intact human X (Fig. 2a). Bands E2, E5, E6, E9 and E10

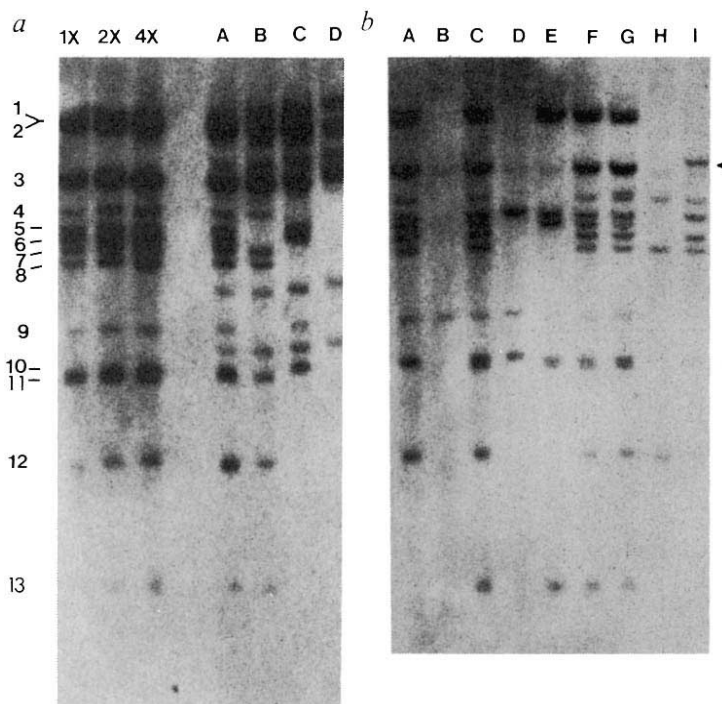


**Fig. 1** A schematic representation of the DXS164 and DXS206 loci. The arrow indicates the position of the t(X;21) translocation exchange point, the zero point for the kilobase scale above. Below the locus diagram is an expansion of a portion of walk clone XJ10 containing subclones XJ10.2 and XJ10.3. The former is a *HindIII*-*SstI* fragment and the latter an *SstI*-*EcoRI* fragment as shown. Both XJ10.2 and XJ10.3 were used as hybridization probes to detect cDNA clones from a human adult muscle cDNA library as described in the text. A resulting 2.0-kb cDNA clone detects 13 *EcoRI* fragments on a genomic Southern blot. These fragments, each containing one or more exons of the gene are indicated on the exon map. The fragments E13 and E11 (marked 13 and 11 on the schematic) map to XJ10.2 and XJ10.3 respectively. No other exons map in the DXS206 locus. The exons on the centromere side (5' end of the gene) are shown in their relative locations as known from deletion analysis (see text) but the distance between them is unknown. The exons mapping in the DXS164 locus are also ordered by deletion analysis, but their location is approximate as the PERT87 clones from the DXS164 locus were not available to us for study. The bottom part of the figure shows some of the deletions that were used to order the exon-containing fragments.



**Fig. 2** *a*, Southern blot showing hybridization of the 2.0-kb cDNA to DNA from an XY male (1X), XX female (2X) and a XXXXY male (4X). Lanes A-C contain DNA from mouse-human somatic cell hybrids: lane A is hybrid AHA11a containing only the human X chromosome; lane B and C are hybrid A2 and B2 containing the der(X) chromosome and, the der(21) chromosome respectively from the t(X;21) translocation patient<sup>23</sup>; lane D is a mouse DNA control sample. *b*, Southern blot showing hybridization of the 2.0 kb cDNA to a series of deletion patients (A-I). The genomic probe indicated is the closest non-deleted probe flanking the deletion. This was determined by probing with pERT87 probes from the DXS164 locus and XJ probes from the DXS206 locus<sup>12,14</sup>. The deletions used to assign exon positions are shown in Fig. 1. The arrow indicates a deletion junction fragment. Deletions involved are: A, HSC 483 (XJ6.2 to pERT87.1); B, HSC 562 (deleted for all of XJ and pERT87); C, HSC 652 (pERT87-15 toward telomere); D, HSC 680 (deleted for all of XJ and pERT87); E, HSC 685 (pERT87-42 toward telomere); F, HSC 705 (no deletion in XJ or pERT87); G, HSC 993 (pERT87-15 toward telomere); H, Guys 163 (pERT87-8 toward centromere); I, Guys 442 (pERT87-1 toward centromere). (Deletions in A-G, see ref. 14; H, I, see ref. 12).

**Methods.** DNA was isolated from lymphoblast cultures or hybrid cultures as previously described<sup>23</sup> and digested with *EcoRI*. Lanes were loaded with 5 µg of DNA for lymphoblasts and 10 µg for hybrid cultures. The samples were electrophoresed on a 0.7% agarose gel and transferred to Hybond N (Amersham) by the method of Southern<sup>24</sup>. The isolated cDNA insert was radiolabelled by hexanucleotide priming<sup>25</sup>. Blots were hybridized in 5×SSPE, 50% formamide and 10% dextran sulphate at 42 °C. The blots were washed with 0.2×SSC at 62 °C.



map to the der(X) and therefore lie on the centromeric side of the translocation junction and the remaining eight fragments map to the der(21) and therefore lie on the telomeric side of the translocation junction (Table 1). This analysis was somewhat complicated by the fact that several mouse bands also hybridize to the cDNA probe (Fig. 2*b*). For example, bands E1 (15 kb), E2 (13.5 kb) and E3 (6.6 kb) are partially obscured by mouse bands that run in a similar position. Definitive assignment of

these bands to the der(X) or der(21) required hybridization with smaller subclones from within the 2.0-kb cDNA (data not shown). The fact that the cDNA clone crossreacts with mouse sequences, at high stringency (Fig. 2*a*) indicates conservation between species. Similar results were obtained with orangutan, hamster, and rabbit in agreement with the data of Monaco *et al.*<sup>13</sup> who first demonstrated sequence conservation in exons from the DMD locus.



To determine which exons map within the DXS206 region shown in Fig. 1, the cDNA clone was hybridized to DNA from each of the XJ phage isolates comprising the DXS206 region. Only one phage clone, XJ10, hybridized to the cDNA and this corresponded to the genomic probe used to obtain the original cDNA clones. Therefore, there are no exons on the centromeric side of XJ10 for a distance of at least 105 kb, and the five *Eco*RI bands that lie on the centromeric side of the translocation junction must lie further than 55 kb from the translocation junction and are outside the DXS206 region.

To extend the mapping of exons outside the DXS206 region, the cDNA clone was hybridized against a series of DNA samples from DMD patients with deletions that had been characterized with genomic probes from the DXS206 and DXS164 locus<sup>12,14</sup>. The genomic extent of some of these deletions is indicated in Fig. 1. A Southern blot of an *Eco*RI digest of DNA from these patients is shown in Figure 2b and the data is summarized in Table 1.

The bands may be ordered on the centromeric side of the translocation junction as: centromere, E9, (E5, E10), (E6, E2) by reference to Table 1. Deletions B, D, H and I extend to the centromeric side, and as E9 is the only band present in deletion B it must be closest to the centromere. Bands E5 and E10 must be next because they are missing from two of these four deletions, and E2 and E6 must be closest to the translocation junction as they are missing from each of these four deletion patients. On the telomeric side the band order is (E4, E8, E12), E7, E1, E3, E13 and E11 by a similar type of analysis. Bands E13 and E11 map in genomic fragments XJ10.2 and XJ10.3 respectively as indicated in Figure 1. In patient I a novel fragment is seen and is interpreted as a junction fragment (J, Table 1) that spans the deletion junction. A schematic diagram of the exon order is presented in Fig. 1.

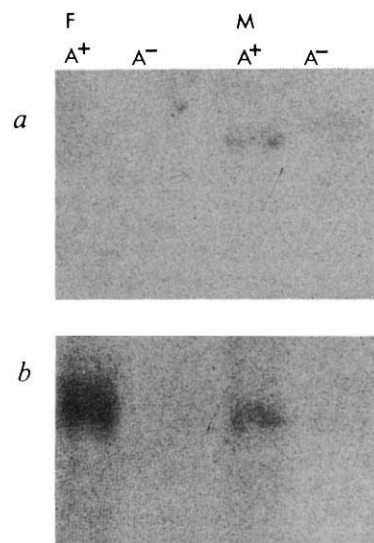
The cDNA clone was also used as a probe on a Northern blot of human adult muscle RNA. A 16-kb transcript was detected in the poly(A)<sup>+</sup> track of muscle (Fig. 3), but was not detectable in the fibroblast messenger RNA preparation. All mRNA samples showed hybridization with a  $\beta$ -hexosaminidase cDNA probe at 2.0 kb, indicating that intact message was present in all mRNA preparations. The 16-kb transcript is of a similar size

**Table 1** Exon mapping using translocation hybrids and deletion patients

| Exon*   | Fragment size (kb) | Translocation chromosome |         | Deletion patients |   |   |   |   |   |   |   |   |   |  |
|---------|--------------------|--------------------------|---------|-------------------|---|---|---|---|---|---|---|---|---|--|
|         |                    | Der(X)                   | Der(21) | A                 | B | C | D | E | F | G | H | I |   |  |
| E8      | 5.3                | +                        | +       | +                 | + | + |   |   | + | + | + | + |   |  |
| E4      | 3.7                | +                        | +       | +                 | + | + |   |   | + | + | + | + |   |  |
| E12     | 0.8                | +                        | +       | +                 | + | + |   |   | + | + | + | + |   |  |
| E7      | 4.0                | +                        | +       | +                 | + | + |   |   | + | + | + | + |   |  |
| E1      | 15                 | +                        | +       | +                 | + | + |   |   | + | + |   |   | J |  |
| E3*(+)  | 6.6                | +                        | +       | +                 | + | + |   |   | + | + |   |   |   |  |
| E13†    | 0.7                | +                        | +       | +                 | + | + |   |   | + | + | + |   |   |  |
| E11†    | 1.9                | +                        | +       | +                 | + | + |   |   | + | + | + |   |   |  |
| t(X;21) |                    |                          |         |                   |   |   |   |   |   |   |   |   |   |  |
| E6      | 4.3                | +                        | +       | +                 | + | + |   |   | + | + | + |   |   |  |
| E2      | 13.5               | +                        | +       | +                 | + | + |   |   | + | + | + |   |   |  |
| E10     | 2.1                | +                        | +       | +                 | + | + | + | + | + | + | + | + |   |  |
| E5      | 4.6                | +                        | +       | +                 | + | + | + | + | + | + | + | + |   |  |
| E9      | 2.6                | +                        | +       | +                 | + | + | + | + | + | + | + | + |   |  |

The size of the *Eco*RI genomic fragments detected by the 2-kb cDNA is shown. The number of each band corresponds to that shown in Fig. 2a, and the bands are ordered in the table according to their relative position from the telomere to the centromere. The fragments that are present in the deletion patient or the hybrids are indicated by a plus sign. The deletions A-I are as described in Fig. 2b; some of these are shown schematically in Fig. 1. J indicates a junction fragment in the deletion.

\* An underlying and weakly hybridizing band is observed when the 6.6 kb band is deleted. This band has not yet been assigned, but appears to map on the centromeric side of the translocation junction.



**Fig. 3** A Northern blot showing hybridization of: *a*, the 2.0-kb cDNA and *b*, control cDNA probe  $\beta$ -hexosaminidase<sup>26</sup> to: F, fibroblast poly(A<sup>+</sup>) RNA and poly(A<sup>-</sup>) RNA; M, muscle poly(A<sup>+</sup>) RNA and poly(A<sup>-</sup>) RNA. Each lane contains 2  $\mu$ g RNA. **Methods.** Total cellular RNA was isolated from cells and tissue by pulverizing under liquid nitrogen, followed by homogenization in guanidinium isothiocyanate and centrifugation through a caesium chloride gradient<sup>27</sup>. Poly(A<sup>+</sup>) RNA was prepared by oligo(dT) cellulose chromatography<sup>28</sup>. The RNA was separated on 1% agarose gels containing formaldehyde<sup>29</sup> and transferred onto Hybond N (Amersham). The cDNA probe was radiolabelled by random hexanucleotide priming<sup>25</sup> to a specific activity of 10<sup>6</sup> c.p.m.  $\mu$ g<sup>-1</sup>. The blots were hybridized in 5 $\times$ SSPE, 50% formamide and 10% dextran sulphate at 42 °C. The blots were washed in 0.2 $\times$ SSC at 55 °C. The size of the messenger RNA was determined using  $\lambda$  HindIII markers.

to that reported by Monaco *et al.*<sup>13</sup>, and there is no reason to suspect that this is not a similar transcript. Our cDNA clone does not extend to pERT87-25, the exon sequence used by Monaco *et al.* to isolate their cDNA clone. Our cDNA includes exons that extend to the region between pERT87-8 and 87-15. The exons in bands E1, E7, E4, E8 and E12 appear to be similar to those previously reported<sup>13</sup> based on our Southern blot analysis of *Hind*III digests (not reported here) and on the *Hind*III map provided by Kunkel (personal communication). Also the *Hind*III digest revealed 3 exons corresponding to E6 and E2, thus one of these fragments must contain two closely spaced exons.

Our cDNA clone therefore hybridizes to a minimum of 13 exons of the DMD/BMD gene. Only two of these are located within the 120 kb of genomic DNA in the DXS206 locus. Six others map within the DXS164 locus and five map into a genomic region of unknown size on the centromeric side of the DXS164-DXS206 locus corresponding to the 5' side of the gene. The eight exons mapping within DXS164-DXS206 span a genomic region of >200 kb including one intron of >105 kb. To our knowledge, this intron is the largest yet reported for any gene. The data is consistent with a gene of megabase proportions as suggested initially by Monaco *et al.*<sup>13</sup> and as supported by pulsed field gel analysis<sup>16-18</sup>, by the wide distribution of translocation breakpoints within Xp21 (refs 4, 19), by the size and complexity of the deletions studied to date<sup>10-12</sup> and by the high frequency of recombination with intragenic probes<sup>20,21</sup>.

Our results constitute definitive evidence that the DXS206 locus surrounding the t(X;21) translocation exchange point is within the gene responsible for Duchenne and Becker muscular dystrophy. This complements and extends similar results from Kunkel's laboratory with the adjacent DXS164 locus. In the

t(X;21) translocation patient, sequence analysis by Bodrug in our laboratory has revealed a small deletion of ~70 bp at the site of exchange<sup>22</sup> but no exons are deleted. The dystrophic phenotype can be entirely accounted for by the fact that the translocation breaks through an intron of the gene separating a few exons at the 5' end from the remainder of the gene. Non-random X-inactivation of the normal X chromosome<sup>3</sup> results in a manifesting heterozygote. With the exception of the oncogene rearrangements in cancer cells, this is the first documented case of a translocation that splits a gene causing a genetic disease.

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## Exchange of terminal portions of X- and Y-chromosomal short arms in human XX males

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In most human 'XX males'<sup>1</sup>, DNA sequences normally found on Yp, the short arm of the Y chromosome, are present on Xp, the short arm of the X chromosome<sup>2-6</sup>. To establish whether this transfer involves a terminal portion of Yp, and whether a terminal portion of Xp is lost in the process, we followed the inheritance of pseudoautosomal restriction fragment length polymorphisms in two XX-male families. One XX male apparently inherited the entire pseudoautosomal region of his father's Y chromosome and no part of the pseudoautosomal region of his father's X chromosome. The second XX male also inherited the entire pseudoautosomal region of his father's Y, but in addition inherited a proximal portion of the pseudoautosomal region of his father's X. These findings argue that XX males result from the transfer of a terminal portion of Yp onto Xp in exchange for a terminal portion of Xp (ref. 7). This implies that the testis-determining factor gene (*TDF*) maps distally in the strictly sex-linked portion of Yp, near the pseudoautosomal domain. The XX males described here appear to result from single (and, at least in the second case, unequal) crossovers proximal to the pseudoautosomal region on Yp and proximal to or within the pseudoautosomal region on Xp.

In man, the most distal portions of Xp and Yp are 'pseudoautosomal'. They undergo frequent X-Y recombination during normal male meiosis. As a result, these portions of Xp and Yp are homologous, and their inheritance is not strictly sex-linked<sup>8-12</sup>.

The *MIC2* gene maps proximally in the pseudoautosomal region, recombining with sex phenotype at a frequency of only 2% in male meiosis<sup>12</sup>. Plasmid pDP1001, which detects a restric-

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tion fragment length polymorphism (RFLP) at *MIC2*, was hybridized to DNAs from the family of XX male LGL163 (Fig. 1a). The father is heterozygous; his 1.4- and 2.0-kilobase (kb) fragments must be on his Y chromosome, because that allele is present in the grandfather but not the grandmother. The XX male inherited the father's Y allele. Densitometry reveals that the XX male has one copy of the 3.3-kb allele. This copy must be from the mother, because she is homozygous for that allele. Thus, at *MIC2*, XX male LGL163 inherited his father's Y allele but not his father's X allele.

The second family was typed for a *MIC2* RFLP detected by pDP1002 (Fig. 1b). XX male LGL1358 has two copies of the 2.6-kb allele, for which the father is homozygous, and one copy of the 3.3-kb allele, for which the mother is homozygous. (Compare, in Fig. 1b, the relative intensities of the 2.6- and 3.3-kb fragments in XX male LGL1358 with those in LGL1364 and LGL1362, both presumably normal heterozygotes.) A similar result was obtained with the pDP1001 RFLP: the XX male has two copies of the allele for which the father is homozygous and one copy of the allele for which the mother is homozygous (not shown).

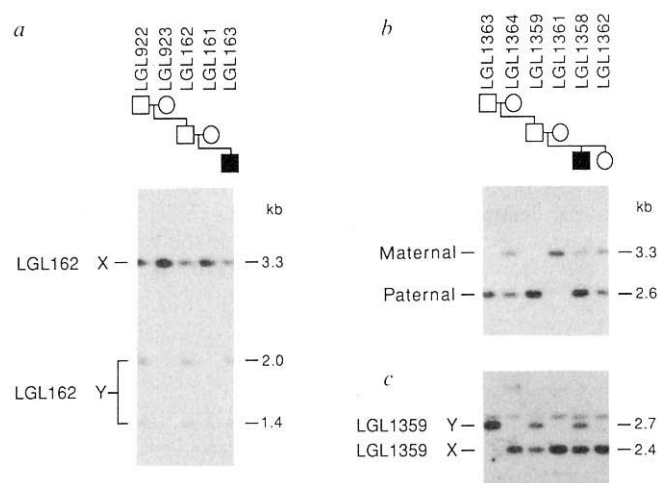
These studies did not reveal the chromosomal origin of the two copies of *MIC2* that XX male LGL1358 received from his father. This information was obtained using an RFLP detected by pSG1, a *MIC2* complementary DNA clone<sup>12</sup>. The father is heterozygous (Fig. 1c). His 2.7-kb fragment must be on his Y chromosome, because that allele is present in the grandfather but not the grandmother. Conversely, the father's 2.4-kb allele must be on his X chromosome. XX male LGL1358 appears to have two copies of the 2.4-kb allele and one copy of the 2.7-kb allele. (Compare, in Fig. 1c, the relative intensities of the 2.4- and 2.7-kb fragments in LGL1358 with those in LGL1359, a normal heterozygote.) The XX male inherited the 2.7-kb fragment from his father's Y chromosome. The XX male presumably inherited one copy of the 2.4-kb fragment from his mother, who is homozygous for that allele. Given the results with pDP1001 and pDP1002, he must have inherited the second 2.4-kb fragment from his father's X chromosome. That is, XX male LGL1358 inherited both his father's X and Y alleles at *MIC2*.

The findings in these XX males have implications for the localization of the testis determining factor gene, *TDF*, on the Y chromosome. An eight-interval deletion map of the Y has



**Fig. 1** Inheritance in two XX male families of RFLPs at *MIC2*. Squares, males; circles, females; filled squares, XX males. Both XX males carry DNA sequences derived from the strictly sex-linked portion of Yp (ref. 3 and unpublished results). Study of the paternal grandparents allows the phase of the pseudoautosomal alleles in the father to be determined. Each autoradiogram lane corresponds to the individual above that lane in the pedigree. *a*, Family 1: probe pDP1001 hybridized to *TaqI*-digested genomic DNAs. Allelic restriction fragments marking the X or Y chromosomes of LGL162, the father, are indicated. *b*, Family 2: pDP1002 hybridized to *TaqI*-digested DNAs. Allelic fragments present in father, LGL1359, or in mother, LGL1361, are indicated. *c*, Family 2: an 0.7-kb *EcoRI*-*StuI* fragment purified from plasmid pSG1 was hybridized to *MspI*-digested DNAs. Fragments that distinguish the X and Y chromosomes of LGL1359, the father, are indicated.

**Methods.** Plasmids pDP1001 and pDP1002 were subcloned from recombinant phages identified by screening a human genomic library with plasmid pSG1, a *MIC2* cDNA clone<sup>21</sup>. Plasmid pDP1001 contains a 1.5-kb genomic *EcoRI*-*PstI* fragment and detects a *TaqI* RFLP with fragments of, in one allele, 3.3 kb and, in a second allele, 1.4 and 2.0 kb. Plasmid pDP1002 contains a 0.8-kb genomic *EcoRI* fragment. It detects an insertion/deletion RFLP (not shown) and a *TaqI* RFLP with allelic fragments of 2.6 and 3.3 kb. These clones were mapped to the region Xp22.32-pter and to the Y by hybridization to DNAs from hybrid somatic cell lines, consistent with their being derived from *MIC2*. Human genomic DNAs were prepared from leukocytes or cultured fibroblasts<sup>22</sup>, digested with restriction endonuclease, electrophoresed on 0.7% agarose gels, and transferred<sup>23</sup> to nylon membrane. Human inserts purified from the plasmids were labelled with <sup>32</sup>P by random-primer synthesis<sup>24</sup>, prehybridized with an excess of sonicated human genomic DNA<sup>25</sup>, and hybridized overnight to genomic DNA transfers at 47 °C in 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM Sodium citrate pH 7.4), Denhardt's (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), 1% SDS, 20 mM NaPO<sub>4</sub> pH 6.6, 50 µg ml<sup>-1</sup> denatured salmon sperm DNA and 10% dextran sulphate. Membranes were washed three times for 15 min each at 65–70 °C in 0.1 × SSC, 0.1% SDS and exposed at –80 °C for 1–3 days with X-ray film backed by an intensifying screen.



been constructed from studies of Y chromosome DNA from XX males and other individuals with sex chromosome anomalies; *TDF* maps to interval 1, on Yp (refs 3 and 13). Uncertainty regarding the order of intervals 1, 2, and 3 on Yp stems from uncertainty as to whether XX males have received terminal or internal portions of Yp (ref. 3). Given that *MIC2* recombines with sex phenotype in only 2% of normal male meioses<sup>12</sup>, the 'terminal' and 'internal' models make opposite predictions as to the inheritance of *MIC2* in XX males. According to the terminal model, an XX male would receive the end of the strictly sex-linked portion of Yp from his father; he would then be very likely to inherit his father's Y-chromosomal allele at the closely-linked *MIC2* locus (probability 98%). According to the internal model, an XX male would not receive the end of the strictly sex-linked portion of Yp; he would inherit his father's Y-chromosomal *MIC2* allele only in the unlikely event of recombination with *MIC2* (probability 2%). That both XX males inherited their father's Y allele at *MIC2* strongly suggests that they received terminal portions of Yp. In turn, this argues that deletion interval 1, containing *TDF*, is just proximal to the pseudoautosomal region. Similarly, that XX male LGL163 did not inherit his father's X allele at *MIC2* suggests that the end of the strictly sex-linked portion of Xp has been lost.

We also followed the inheritance in these families of five other pseudoautosomal RFLPs (Table 1). Three of these polymorphic loci, *DXYS17*, *DXYS15* and *DXYS28*, show partial sex linkage, recombining with sex phenotype in male meiosis at frequencies of about 14%, 32% and 38%, respectively (ref. 11 and D.P. *et al.*, in preparation). The other two loci, *DXYS20* and *DXYS14*, show no detectable sex linkage and map to the most distal portion of the pseudoautosomal region, near the telomeres of Xp and Yp (refs 9 and 11; D.P. *et al.*, in preparation).

All five RFLPs are informative in the first family, and at each locus the XX male inherited from father the Y but not the X allele (Table 1). For example, the father is heterozygous for the *DXYS14* RFLP (Fig. 2a). The father's X and Y alleles are identified by their presence in, respectively, the grandmother and grandfather. XX male LGL163 inherited from his father the Y but not the X allele at *DXYS14*.

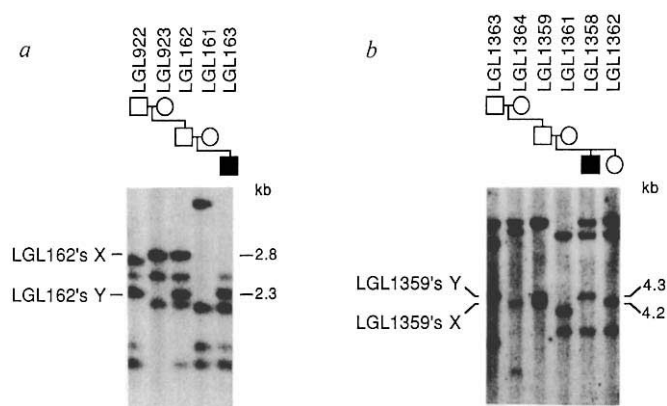
Thus, at six loci, together spanning nearly the entire pseudoautosomal portion of the sex chromosomes, XX male LGL163 inherited from father the Y but not the X alleles. These results argue that LGL163 inherited the pseudoautosomal region of his father's Y chromosome intact and unrecombined. A double crossover between two of the pseudoautosomal markers for which we tested is unlikely, given that double recombinants

**Table 1** X- or Y-chromosomal origin of pseudoautosomal RFLP alleles transmitted from fathers to XX-male sons

| Locus         | Probe   | Recombination<br>with sex phenotype (%) | Reference                              | Allele from father    |                        |
|---------------|---------|-----------------------------------------|----------------------------------------|-----------------------|------------------------|
|               |         |                                         |                                        | LGL163<br>(XX male 1) | LGL1358<br>(XX male 2) |
| <i>DXYS14</i> | 29C1    | 50                                      | 9,11                                   | Y                     | Y                      |
| <i>DXYS20</i> | pDP230  | 50                                      | Page <i>et al.</i><br>(in preparation) | Y                     | NI                     |
| <i>DXYS28</i> | pDP411a | 38                                      | Page <i>et al.</i><br>(in preparation) | Y                     | NI                     |
| <i>DXYS15</i> | 113D    | 32                                      | 10, 11                                 | Y                     | NI                     |
| <i>DXYS17</i> | 601     | 14                                      | 11                                     | Y                     | NI                     |
| <i>MIC2</i>   | pSG1    | 2                                       | 12                                     | Y                     | X and Y                |
|               | pDP1001 |                                         |                                        |                       |                        |
|               | pDP1002 |                                         |                                        |                       |                        |

DNA probes detecting pseudoautosomal RFLPs were hybridized to *TaqI*, *MspI*, or *EcoRI*-digested DNAs from two XX males, their parents, and paternal grandparents. NI, not informative.





**Fig. 2** Inheritance in two XX-male families of RFLPs at *DXYS14*. *a*, Family 1: probe 29C1 was hybridized at 42 °C to *TaqI*-digested genomic DNAs. After washing at 60 °C, the transfer membrane was exposed with X-ray film for 3 days. Probe 29C1 detects a polymorphic family of related sequences, resulting in complex hybridization but simple, single-locus inheritance; a typical allele comprises a collection of restriction fragments<sup>9,11</sup>. Some of the allelic restriction fragments that distinguish the X and Y chromosomes of LGL162, the father, are indicated. *b*, Family 2: 29C1 hybridized to *EcoRI*-digested DNAs. Some of the allelic restriction fragments marking the X and Y chromosomes of LGL1359, the father, are indicated.

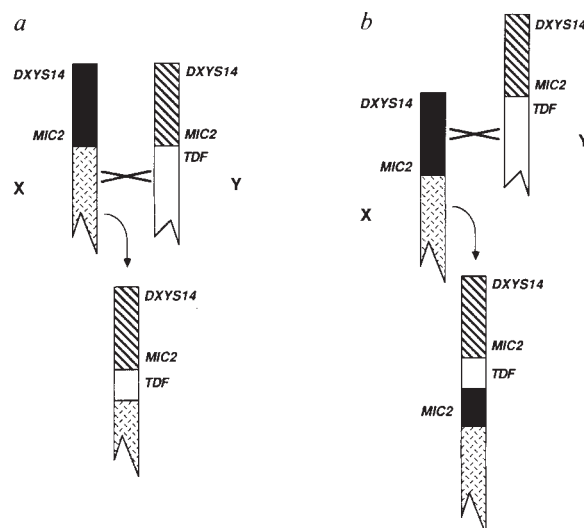
occur rarely if ever within the pseudoautosomal region in man (ref. 11; D.P. *et al.*, in preparation). The results also suggest that XX male LGL163 inherited no part of the pseudoautosomal region of his father's X chromosome.

In the second family, apart from *MIC2*, one pseudoautosomal locus was informative (Table 1). At the distal locus *DXYS14*, XX male LGL1358 inherited from his father the Y allele but not the X allele (Fig. 2*b*).

We propose that sex reversal in these XX males is the result of aberrant Xp-Yp exchanges (Fig. 3). XX male LGL163 appears to have inherited the entire pseudoautosomal region of his father's Y chromosome but no part of the pseudoautosomal region of his father's X chromosome. This is probably the result of a single crossover proximal to *MIC2* on Xp and proximal to *TDF* on Yp (Fig. 3*a*). The result is a transfer of a terminal, male-determining portion of Yp onto distal Xp, in exchange for a terminal portion of Xp, as predicted by the 'X-Y interchange' model<sup>7,14</sup>.

XX male LGL1358 also seems to have inherited the entire pseudoautosomal region of his father's Y chromosome. In addition, however, he inherited the proximal portion (*MIC2*) but not the distal portion (*DXYS14*) of the pseudoautosomal region of his father's X chromosome. This is probably the result of a single crossover in the pseudoautosomal region (between *MIC2* and *DXYS14*) on Xp and proximal to *TDF* on Yp (Fig. 3*b*), as has been hypothesized to occur in some XX males<sup>14</sup>.

X-Y exchanges like that in Fig. 3*b* produce XX males with two copies of all strictly X-linked loci. In contrast, exchanges of the sort seen in LGL163 (Fig. 3*a*) might produce XX males with a single copy of strictly sex-linked loci on distal Xp. This may explain why some XX males express their fathers' alleles for Xg, a dominant, X-linked marker on distal Xp, whereas most, including LGL163, do not<sup>1,15</sup>. Exchanges like that in Fig. 3*b* would yield *TDF*-bearing X chromosomes whose length is greater than that of normal X chromosomes. Depending on the positions of Xp and Yp breakpoints, exchanges like that in Fig. 3*a* might yield abnormally long or short *TDF*-bearing X chromosomes. Such alterations may explain the observation that, in many XX males, one of the two X chromosomes seems to be abnormally long<sup>16</sup> or has an altered high-resolution banding pattern<sup>17</sup>.



**Fig. 3** Genesis of *TDF*-bearing X chromosomes in XX males by single crossovers between Xp and Yp during or prior to paternal meiosis. Differentially shaded regions depict the pseudoautosomal (black and striped) and strictly sex-linked (stippled and white) portions of Xp and Yp. On Yp, the point of crossing over is proximal to *TDF*, which in turn is proximal to the pseudoautosomal region. On Xp, crossing over can occur either *a*, proximal to *MIC2*, perhaps in the X-specific region, or *b*, distal to *MIC2*, in the pseudoautosomal region. Unequal crossing-over depicted in *b* produces an X-Y interchange product carrying two copies of *MIC2*.

Distal portions of Xp and Yp pair during male meiosis<sup>18,19</sup>. Does this pairing contribute to the rather high frequency (1 in 20,000 males<sup>1</sup>) at which XX males occur? Various mechanisms can be envisaged. First, homologous X-Y recombination initiated in the pseudoautosomal region might, on occasion, give rise to branch migration into the strictly sex-linked portions of the sex chromosomes, with resolution proximal to *TDF*. This seems unlikely, however, because branch migration seems to require that recombining chromosomes have very similar DNA sequences; the Yp-specific DNA sequences that XX males inherit are not homologous to Xp.

A second possibility is more likely. The Xp-Yp synaptonemal complex extends far beyond the pseudoautosomal region, involving the distal quarter of Xp and virtually all of Yp (ref. 20). This synapsis might occasionally produce exchanges between the strictly sex-linked portions of the X and Y chromosomes. These exchanges may or may not be legitimate recombination events occurring at sites of limited X-Y homology. To account for XX males in whom the point of recombination on X is pseudoautosomal, one would have to suppose that Xp-Yp synapsis is compatible with the pseudoautosomal portions of X and Y chromosomes being grossly out of register. Perhaps the pseudoautosomal regions of the X and Y chromosomes, so highly recombinogenic in male meiosis, retain this property when misaligned.

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## Borders of parasegments in *Drosophila* embryos are delimited by the *fushi tarazu* and *even-skipped* genes

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One of the earliest molecular signs of segmentation in *Drosophila* embryos is the striped expression of some pair-rule genes<sup>1</sup> during the blastoderm stage. Two of these genes, *fushi-tarazu* (*ftz*) and *even-skipped* (*eve*) are expressed during this stage in complementary patterns of seven stripes<sup>2-8</sup> which develop and disappear in concert<sup>8</sup>. Here, we map the cells expressing each of these two pair-rule genes with respect to the 14 stripes of cells expressing the *engrailed* gene. We find that both *ftz* and *eve* generate stripes which have sharp boundaries at the anterior margin, but fade away posteriorly. The anterior boundaries correspond cell by cell with the anterior boundaries of expression of the *engrailed* gene. We therefore suggest that a key function of early *ftz* and *eve* gene activity is the formation of a sharp stable boundary at the anterior margin of each stripe. These boundary lines, rather than the narrowing zonal stripes, would delimit the anterior boundaries of *engrailed* and other homoeotic genes and thereby subdivide the embryo into parasegments<sup>9</sup>.

The *engrailed* gene product was mapped directly by staining embryos for binding of an antibody<sup>10</sup> against the engrailed product ('anti-engrailed' antibody). The embryos we used contained constructs consisting of the promoter of either the *ftz* gene<sup>4</sup> or the *eve* gene joined to the  $\beta$ -galactosidase gene from *Escherichia coli*. The patterns of expression of the *ftz* and *eve* genes were detected using monoclonal antibodies against  $\beta$ -galactosidase. The *engrailed* gene product is seen in the nuclei and the  $\beta$ -galactosidase in the cytoplasm; this allows us to score single cells independently for both proteins. Compared with the native *ftz* and *eve* products  $\beta$ -galactosidase seems to be relatively stable<sup>4,11</sup>; hence, the earlier activity of both the *ftz* and *eve* promoters in particular cells is signalled by the presence of  $\beta$ -galactosidase which continues to accumulate in those cells or in their descendants.

During the entire period when the germ band is extended, and also later when the germ band has shortened<sup>12</sup>, the *engrailed* gene is expressed in nuclei which are arranged in sharply demarcated stripes that constitute the posterior compartments of the ectoderm<sup>10,13-15</sup>. For *ftz*, the  $\beta$ -galactosidase staining is found only in even-numbered parasegments and is graded in intensity,

**Fig. 1** *a*, Lateral view of embryo with germ band extended (stage 11, ref. 12). Numbers indicate the *engrailed* (grey nuclei) stripes of particular parasegments<sup>9</sup>. Cytoplasmic staining for *ftz*- $\beta$ -galactosidase is brown, and marks the anterior regions of even-numbered parasegments. *b*, Section of same embryo shown in Fig. 1. The section gives a higher resolution. *c*, Glancing section of dorsal posterior region of an extended germ band embryo to show precise coextension of *engrailed* (greyish nuclei) and *ftz*- $\beta$ -galactosidase (brown cytoplasm) expression. This is particularly clear at the anterior border of parasegment 14 (arrow). Note that the 15th *engrailed* stripe<sup>37</sup> is exceptional and falls within the broader posterior *ftz* stripe. Bright field. *d*, Lateral glancing section of embryo with germ band shortened (stage 13, ref. 12). The anterior boundaries of the parasegments are visible on the surface, behind them cells dip out of the plane of section into the segmental grooves. Note, at much of the anterior boundaries of the even-numbered parasegments, the *engrailed* expression (greyish nuclei) and the *ftz*- $\beta$ -galactosidase (brown cytoplasm) are clearly coextensive (arrows). In each even-numbered stripe there is a small ventral patch of  $\beta$ -galactosidase staining that extends anteriorly. We do not know what this signifies. *e*, Another picture of the section in Fig. 3 (Nomarski interference contrast microscopy).

**Methods.** For immunohistochemistry, embryos were fixed in 4% paraformaldehyde in heptane/methanol<sup>38</sup>, treated with anti-engrailed antibody overnight (dilution 1:100), then with biotinylated secondary antibody and stained with Vectrolabs Vectastain ABC kit using diaminobenzidine; makers instructions were followed except that 30  $\mu$ l of 1% nickel sulphate and 1% cobalt chloride in water were added to 1 ml staining mix. This gave a slate grey colour<sup>39</sup>. Embryos were then treated with anti- $\beta$ -galactosidase antibody (C. Doe and C. Goodman) (dilution 1:100), biotinylated secondary antibody and stained as above except the metal ions were omitted. This gave an orange-brown colour. Individual embryos were mounted on slides and photographed in various orientations, removed, embedded in agar, oriented and embedded in araldite. Thick sections (20-40  $\mu$ m) were cut, mounted and photographed. These photographs are the best we were able to prepare to show what is easily seen down the microscope simply by focusing up and down. In sections and whole mounts there is a trade-off between colour intensity and resolution. For immunofluorescence, embryos fixed as above were incubated with the anti-engrailed and anti- $\beta$ -galactosidase primary antibodies, washed and incubated in both fluorescein isothiocyanate (FITC)-linked-anti-rabbit and rhodamine isothiocyanate (RITC)-linked-anti-mouse secondary antibodies. The *eve*- $\beta$ -galactosidase hybrid gene consists of 6.3 kilobases (kb) of the *eve* 5' flanking sequence extending into the structural gene to the *Ava*II site in codon 22 of the *eve* coding sequence, fused with the  $\beta$ -galactosidase structural gene. To ensure consistent polyadenylation of the fusion gene transcript, 800 base pairs (bp) of the 3' flanking region of the tubulin  $\alpha$ 1 gene<sup>40</sup> (beginning at the *Scal* site at position 1,960 and including the poly(A) addition site at position 2,185) was fused 3' to the stop codon of the  $\beta$ -galactosidase coding sequence. The *eve*- $\beta$ -galactosidase fusion gene was inserted into the Carnegie 20 vector and incorporated into the genome by P-element mediated transformation. Flies used in this study were homo- or hemizygous for a single insertion on the X chromosome. We cannot be certain that either the *ftz* or *eve*- $\beta$ -galactosidase gene fusions are expressed exactly like the native *ftz* and *eve* genes. However, within the limits of resolution of previous studies, the major features of expression, notably the seven-striped zebra patterns, appear very similar, if not identical. Both the *ftz* and *eve* genes are actually expressed at a low level throughout much of the syncytial blastoderm and the *eve* gene also generates seven additional minor stripes after the onset of gastrulation<sup>8</sup>. We could not detect these additional aspects of *ftz* and *eve* expression possibly because they are too ephemeral or insubstantial. Hence our analysis is limited to the prominent seven stripes of both genes.

starting abruptly at the anterior margin of the stripe and fading away gradually in the more posterior cells. The pattern of *eve*- $\beta$ -galactosidase expression is similar, except that the stripes are restricted to the odd-numbered parasegments. At the anterior margins the expression of *engrailed* and  $\beta$ -galactosidase are precisely coextensive (Figs 1-3). Later stages show the same pattern but the *ftz* and *eve* stripes have narrowed further and the stain is concentrated in the anterior parts of the parasegment (Fig. 1*d*). The anterior borders of *engrailed* and *ftz* expression also coincide (S. Carroll and S. DiNardo, personal communication).

We have not been able to map *ftz*- $\beta$ -galactosidase or *eve*- $\beta$ -



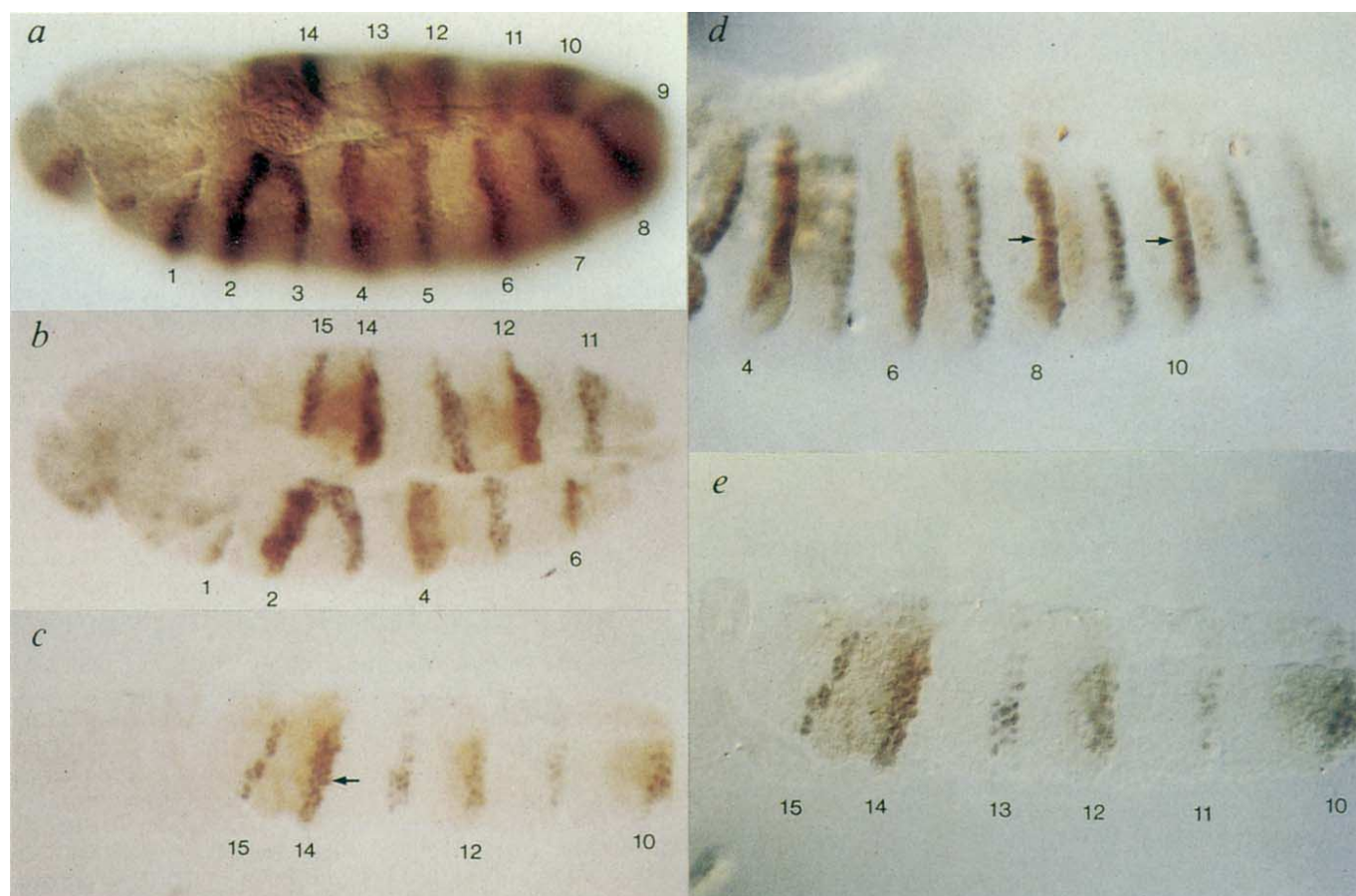


Fig. 1 (Legend facing)

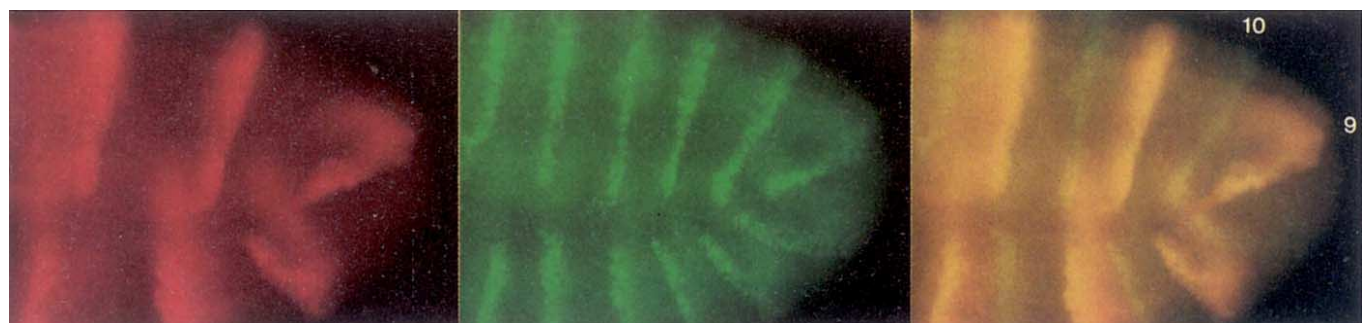


Fig. 2 Three views of an extended germ band to show expression of *eve*- $\beta$ -galactosidase (red cytoplasm) *engrailed* (green nuclei) and the superposition of both.

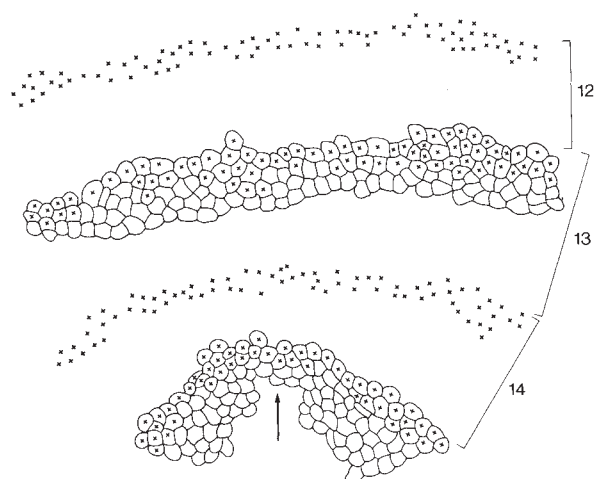
galactosidase expression in the stripes at blastoderm as our methods are not sufficiently sensitive, so we cannot be certain where these two genes would have been expressed at that stage. The earliest we have seen both signals is at the onset of gastrulation (stage 7, ref. 12) when, although the colours are too pale to photograph, the pattern is clearly the same as later; that is, sharp coextensive boundaries at the anterior edges of the *ftz* and *engrailed*, and *eve* and *engrailed*, stripes, and no definable posterior boundaries. We believe this pattern is the direct result of the same pattern at the blastoderm stage; indeed, Carroll and DiNardo (unpublished observations) have observed that, in parasegment 2 where the *engrailed* product is first detectable, the *engrailed* and *ftz* stripes are coextensive at their anterior margins. All these points suggest, but do not prove, that even from the blastoderm stage, the anterior border of the stripe is stable, but the posterior one is not.

By definition, the parasegmental border should coincide with the anterior margins of the *engrailed* stripes<sup>9</sup>. Consequently, assuming that the *ftz*- $\beta$ -galactosidase and *eve*- $\beta$ -galactosidase hybrid genes are expressed like the native genes, their anterior borders of expression must also be the anterior borders of the even- and odd-numbered parasegments, respectively. This finding partially fulfils the prediction<sup>4,16</sup> that the *ftz* stripes would prove to be parasegmental.

The stripes of both the RNA and protein products of *ftz* and *eve* seem to narrow continuously from when they are first established during the blastoderm stage until they disappear following germ band extension<sup>2,3,7-9</sup>. Our observation that the stripes of  $\beta$ -galactosidase expression have sharp boundaries at only the anterior margins suggests that the native RNA and protein stripes narrow exclusively from the posterior edge.

The development of alternate *engrailed* stripes does not occur





**Fig. 3** Camera lucida drawing of *eve*- $\beta$ -galactosidase and *engrailed* expression following germ band extension (dorsal aspect). Numbers denote parasegments. Nuclei labelled by the *engrailed* antibody are indicated by the X over the centre of each. Labelled cell boundaries could be easily seen and drawn at the anterior margin of each stripe but as the rhodamine intensity declines more posteriorly, the cell outlines become too faint to draw accurately; thus fluorescence extends more posteriorly in each stripe than indicated. Note that the most posterior  $\beta$ -galactosidase stripe and the 15th *engrailed* stripe<sup>31</sup> are coextensive at their anterior boundaries; these are immediately behind parasegment 14, and both develop later than the stripes which demarcate parasegments 1–14. Arrow, midline. We follow the Nüsslein-Volhard convention: anterior is to the left and, where appropriate, ventral to the bottom.

in *ftz* mutant embryos<sup>17</sup>, whereas *ftz* expression is normal in *engrailed* mutant embryos<sup>7,18</sup>. Similarly, *eve* expression begins normally in *engrailed* mutant embryos<sup>7</sup>, whereas *engrailed* expression is largely eliminated in *eve* mutant embryos<sup>7,8</sup>. Thus, the *ftz* and *eve* gene functions appear to delimit the boundaries of *engrailed* and not vice versa.

The patterning of the *ftz* and *eve* stripes depends on positional information derived from the oocyte<sup>19–21</sup>, and the cell-by-cell response to that information<sup>22</sup> defines exactly which cells will belong to one parasegment, and which will belong to the adjacent ones. Because the pattern of cells on the surface of the blastoderm is not fixed (in the sense that every cell is defined with an exactly equivalent cell in each individual) it follows that there will be variation and imprecision. Thus, however precise the positional mechanism of drawing the line may be, there will inevitably be some wiggle in the border as it is drawn, as has been observed for *ftz* and *eve* (ref. 3 and Fig. 3). It is necessary that this wiggle in the lines should be respected by the sets of selector genes which are activated later. For example, in the blastoderm near the presumptive anterior border of parasegment 6, all cells which will belong to parasegment 6 will express *ftz* and should also be positive for expression of both *engrailed* and *Ultrabithorax* (*Ubx*). If this were not achieved, individual cells could 'belong' to parasegment 6 with respect to *Ubx* and to parasegment 5 with respect to *engrailed*; these cells might give rise to clones with the wrong instructions which would develop inappropriately<sup>23</sup>. Thus, once the border is defined by *ftz* or *eve*, however wiggly, the other later acting genes must respect exactly the same border and this can only be achieved by interactions between genes within single cells. Later, because of the effect the *engrailed* product has on the affinities of posterior cells, the wiggly border can become straightened<sup>13,24</sup>.

It has been argued that the *ftz* gene is required for activation of selector genes, being a linchpin linking segmentation itself with diversification of parasegments<sup>16,17,25</sup>. Our results, which

point to the function of *ftz* and *eve* in locating the boundaries of selector gene expression, are consistent with this view and indicate the nature of the link. Thus, the same lines drawn by the anterior boundaries of *ftz* and *eve* stripes link segmentation (they define the compartment boundaries) with diversification (they delimit the expression of selector genes such as *Ubx*). Moreover, segmental fields or gradients of positional information are probably set up with reference to limits which may coincide with compartment boundaries<sup>26–28</sup>. The special properties of such boundaries, which can act as barriers to the intercellular passage of molecules or signals<sup>29–33</sup>, as well as positional information itself, may be important in locating selector gene expression.

In summary, we propose that a main function of periodic *ftz* and *eve* expression is to divide the embryo into parasegments by defining boundary lines (interfaces between populations of cells), not domains or stripes. It may even be immaterial which cells express these pair-rule genes away from the anterior borders of the stripes—a possibility which fits with the progressive narrowing of the stripes during development and the lack of any stable boundary at their posterior limits. This model differs from those combinatorial theories which propose that the pair-rule genes distinguish all the cells in the stripes from those in the interstripes<sup>16,34–36</sup>.

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## Two zinc fingers of a yeast regulatory protein shown by genetic evidence to be essential for its function

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The best-understood protein structure involved in DNA binding is the helix–turn–helix motif<sup>1</sup>. A second DNA-binding domain, the finger structure, has been proposed on the basis of sequence analysis<sup>2,3</sup>, partial proteolysis<sup>2</sup> and zinc content<sup>2</sup> of *Xenopus* transcription factor TFIIIA. Other eukaryotic proteins were subsequently found to contain contiguous repeat units of the postulated finger motif<sup>4–6</sup>. Each repeat unit contains thirty amino acids and is thought to bind a zinc atom using two cysteines and two histidines as ligands. The protein loop or finger between apparent zinc ligands is rich in DNA-binding residues and is thought to make specific contacts with DNA<sup>2</sup>. The yeast protein ADR1, a positive regulator of transcription of the gene *ADH2*, contains two finger domains in a region of the protein required for transcriptional activation<sup>6</sup>. Nineteen independently isolated *adr1* mutations induced by hydroxylamine were found at nine different amino-acid positions, seven of which are in the two finger domains. All four mutations that altered invariant cysteine or histidine residues led to an *adr1* null phenotype. Only one other mutation caused an *adr1* null phenotype. Thus, one finger domain is not sufficient for ADR1 activity. This provides the first evidence that, as is consistent with the proposed model, the invariant cysteine and histidine residues are essential for the formation of the finger structure.

We isolated *Adr1*<sup>–</sup> mutants by taking advantage of an unusual property of an ADR1– $\beta$ -galactosidase ( $\beta$ -gal) fusion protein: it activates transcription of the normally glucose-repressible *ADH2* gene in a partially constitutive manner<sup>7</sup> (see Fig. 1 legend for details). A second advantage of using the ADR1– $\beta$ -gal fusion protein was the ability to select *Adr1*<sup>–</sup> mutants and subsequently screen for those that were  $\beta$ -gal<sup>+</sup> and therefore due to missense mutations. There are two classes of *Adr1*<sup>–</sup> mutants based on their level of *ADH2* expression (Table 1). The first class contain mutations in the finger domain and did not express *ADH2* under either repressing or derepressing growth conditions. The second class (mutants 1, 23, 2, 26, 21, 16) express *ADH2* poorly during glucose growth but derepressed *ADH2* to a level 10–20% of that observed for 521-6- $\Delta$ 1 carrying the unmutagenized plasmid YCp642ADR1-lacZ. All transformants had approximately the same amount of  $\beta$ -gal activity as strain 521-6- $\Delta$ 1 carrying the unmutagenized plasmid, about 5 U mg<sup>–1</sup> (data not shown). This indicates that the mutations do not affect expression of ADR1-lacZ and suggests that they are in the ADR1 coding sequence.

The possibility that the mutant phenotype resulted from mislocalization of *adr1*– $\beta$ -gal fusion proteins was tested using the  $\beta$ -gal moiety as a tag<sup>8,9</sup>. The mutant *adr1*– $\beta$ -gal fusion proteins were localized to the nucleus (Fig. 2a–d shows the results obtained with two representative mutants). These results indicate that the *Adr1*<sup>–</sup> mutants are not defective in nuclear targeting or retention and suggest that they contain full-length fusion proteins.

Results of DNA sequence analysis of nineteen *adr1* mutations are shown in Table 1. These mutations led to amino-acid changes at nine different positions in the amino-terminal 150 amino acids of the ADR1 polypeptide. Eighteen mutations resulted in single amino-acid changes whereas mutation 21 (Thr 70, Thr 71  $\rightarrow$  Ile 70, Ile 71) had three base substitutions that resulted in two adjacent amino-acid changes.

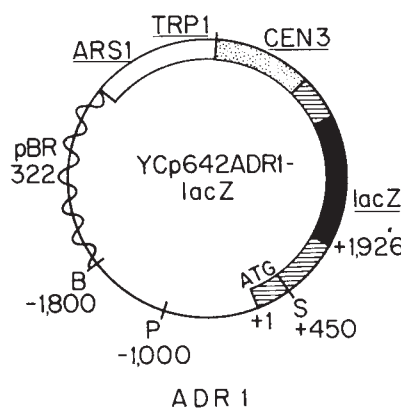


Fig. 1 Map of plasmid YCp642ADR1-lacZ, which contains a yeast centromere (*CEN3*, stippled), *TRP1* for selection in yeast, *ARS1* for plasmid replication in yeast, *pBR322* (wavy line) for replication and selection in *E. coli* and *ADR1-lacZ* (hatched, *ADR1*; solid, *lacZ*). Restriction sites: B, *Bam*HI; P, *Pvu*II and S, *Sph*I. Numbering is in bp and relative to the A of the ATG translational initiation codon as +1. The *ADR1-lacZ* fusion encodes the amino-terminal 642 amino acids of *ADR1* fused to an *E. coli lacZ* gene lacking its first seven codons<sup>19</sup>. The entire *ADR1* gene could encode a protein containing 1,323 amino acids<sup>6</sup>. Fusion of  $\beta$ -gal to the first 642 amino acids of *ADR1* has the same effect as overexpressing *ADR1*; both result in *ADH2* expression during glucose growth conditions<sup>7</sup>. Strain 521-6- $\Delta$ 1 (*MATa adh1-11 ADH2 adh3 adr1- $\Delta$ 1::LEU2 trp1 ura1 leu2*) carrying YCp642ADR1-lacZ is allyl alcohol-sensitive (see below) and Antimycin A-resistant.

**Methods.** Hydroxylamine mutagenesis of plasmid DNA ( $\mu$ g) was performed as previously described<sup>15</sup>. Strain 521-6- $\Delta$ 1 was transformed with the mutagenized plasmid and transformants were selected on plates containing yeast minimal media, 20 mM allyl alcohol, an amino-acid mixture lacking tryptophan and 2% glucose. This selects for transformants which carry the YCp642ADR1-lacZ plasmid, yet synthesize small amounts of *ADH2* activity<sup>20</sup>. Most transformants were defective in *ADH* activity as indicated by their inability to grow on plates containing 1 p.p.m. antimycin A (ref. 18). Approximately 10% of the allyl alcohol-resistant transformants were  $\beta$ -gal<sup>+</sup> as determined by a nitrocellulose filter  $\beta$ -gal assay<sup>21</sup> using the chromogenic substrate Xgal. Twenty six allyl alcohol-resistant,  $\beta$ -gal<sup>+</sup> mutants were characterized. Analysis of nineteen mutants is presented here; the other six mutants either were antimycin A-resistant and wild-type for *ADH2* expression or had plasmid rearrangements. To demonstrate that the mutations are plasmid-linked, Plasmid DNA was isolated from yeast<sup>17</sup> and used to transform *E. coli* to ampicillin resistance. Plasmid DNA was isolated from *E. coli*<sup>22</sup> and used to retransform strain 521-6- $\Delta$ 1. Two independent transformants carrying plasmid DNA derived from each mutant were analysed by allyl alcohol and antimycin A plate assays and by *ADH* enzyme assays. The results of *ADH* enzyme assays (Table 1) indicated that the mutant phenotype was conferred by the plasmid. The region of *ADR1* in these plasmids which carries the mutation was determined by removing the 2.25-kilobase (kb) *Bam*HI–*Sph*I restriction fragment from these plasmids and cloning it in place of the corresponding wild-type fragment in plasmid YEp505ADR1 (ref. 6). Transformants carrying these plasmids were tested by antimycin A plate assays and by *ADH* enzyme assays and were found to have a phenotype similar to the original mutant strains (data not shown). This indicates that the mutation is in the 2.25-kb *ADR1* fragment and maps 5' to the *Sph*I site in the *ADR1* coding sequence.

Figure 3a shows that all the *adr1* mutations are clustered in a small region of the *ADR1* gene. Seven of the nine amino-acid positions altered were in the finger domain of ADR1. The other two mutations, Thr 70, Thr 71  $\rightarrow$  Ile 70, Ile 71 and Pro 87  $\rightarrow$  Leu, led to amino-acid changes on the amino-terminal side of the finger domain. This demonstrates that a region outside the finger domain is essential for normal ADR1 function. It is striking that no mutations were recovered in residues 160–642, on the



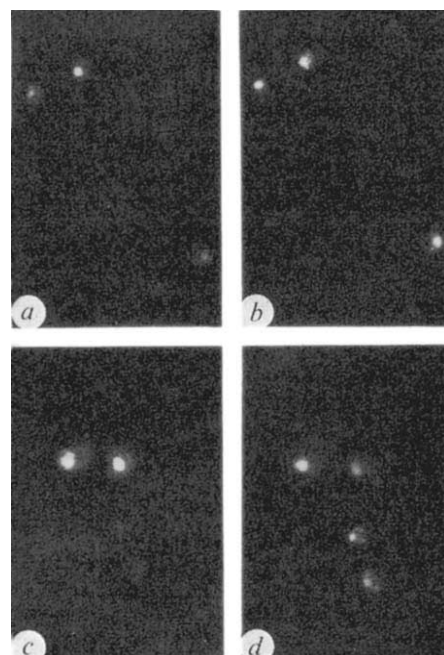
**Table 1** Characterization of *adr1* mutations

| Isolate number             | Alteration                                          | ADH activity<br>repressed | ADH activity<br>derepressed | Antimycin-A<br>phenotype |
|----------------------------|-----------------------------------------------------|---------------------------|-----------------------------|--------------------------|
| <b>Finger mutations</b>    |                                                     |                           |                             |                          |
| 4, 7, 11, 18, 19, 22, 24   | Cys 106(TGC) → Tyr(TAC)                             | <5                        | <20                         | —                        |
| 6, 8, 14                   | Cys 109(TGT) → Tyr(TAT)                             | <5                        | <20                         | —                        |
| 1, 23                      | Ala 114(GCA) → Val(GTA)                             | 10                        | 1,200                       | + / —                    |
| 3                          | His 118(CAC) → Tyr(TAC)                             | <5                        | <20                         | —                        |
| 25                         | His 122(CAT) → Tyr(TAT)                             | <5                        | <20                         | —                        |
| 5                          | Cys 134(TGT) → Tyr(TAT)                             | <5                        | <20                         | —                        |
| 2, 26                      | Thr 142(ACT) → Ile(ATT)                             | <5                        | 1,000                       | —                        |
| <b>Other mutations</b>     |                                                     |                           |                             |                          |
| 21                         | Thr 70(ACC), Thr 71(ACA) → Ile 70(ATT), Ile 71(ATA) | 10                        | 1,600                       | +                        |
| 16                         | Pro 87(CCC) → Leu(CTC)                              | 10                        | 1,800                       | + / —                    |
| <b>Controls</b>            |                                                     |                           |                             |                          |
| No plasmid                 |                                                     | <5                        | <20                         | —                        |
| Wild-type <i>ADR1-lacZ</i> |                                                     | 600                       | 8,000                       | ++                       |
| Wild-type <i>ADR1</i>      |                                                     | <5                        | 2,000                       | —                        |

DNA for sequence analysis was derived from plasmid YCp642adr1-*X-lacZ* where *X* is the mutant isolate number. *EcoRI-SphI* restriction fragments from this plasmid corresponding to nucleotide positions +55 to +450 in *ADR1* (relative to the ATG) were cloned into the replicative form of M13mp18 and M13mp19 phages<sup>13</sup>. *PvuII-EcoRI* fragments corresponding approximately to positions -1,000 to +54 in *ADR1* were cloned into M13mp19. JM103 was used as a host strain for transformation by recombinant DNA molecules<sup>13</sup>. Sequence analysis was performed by the dideoxy chain termination method<sup>14</sup>. DNA from each isolated mutant was sequenced on both strands for the *EcoRI-SphI* fragments. Only C-G → T-A transition mutations were found, as expected for hydroxylamine-induced mutations<sup>15</sup>. The mutational analysis presented here does not address the importance of the conserved hydrophobic residues in *ADR1*. Codons that encode these tyrosine, phenylalanine and leucine residues cannot be mutated to encode other amino acids by hydroxylamine. Neither nonsense nor frameshift mutations were found, consistent with the  $\beta$ -gal<sup>+</sup> phenotype of the mutant strains. DNA sequence was derived from one strand of DNA for the *PvuII-EcoRI* fragment that corresponded to the first 54 base pairs (bp) of *ADR1* coding sequence. Wild-type sequence data was obtained in all cases and sequencing was performed for one representative mutation for each altered amino-acid position except Cys 109 → Tyr, His 118 → Tyr and Thr 70, Thr 71 → Ile 70, Ile 71. Strain 521-6- $\Delta 1$  (*MATa adh1-11 ADH2 adh3 adr1- $\Delta 1$ : :LEU2 trp1 ura1 leu2*) was transformed with plasmid YCp642ADR1-lacZ or its mutant derivatives by the lithium acetate procedure selecting for a Trp<sup>+</sup> phenotype<sup>16</sup>. The plasmid DNA was isolated from the original allyl alcohol-resistant, Trp<sup>+</sup> transformants (see Fig. 1 legend) and passaged through *Escherichia coli*. A map of the plasmid is given in Fig. 1. Cells were grown in yeast minimal media containing 5% glucose and supplemented with an amino-acid mixture lacking tryptophan for repressed growth or yeast complete media containing 3% glycerol and 1% ethanol for derepressed growth. Cell extracts were used for ADH assays<sup>17</sup> as previously described. Assays were done in duplicate for at least two independent transformants. ADH activities are given in mU mg<sup>-1</sup> and are given as the means of the total number of assays. Assay numbers reflect only ADHII and are  $\pm 30\%$ . Cells must synthesize ADH and ferment to grow in the presence of the respiratory inhibitor antimycin A<sup>18</sup>. Most *Adr1*<sup>-</sup> mutants did not grow on media containing glucose and antimycin A. *Adr1*<sup>-</sup> mutants 1, 16, 21 and 23 grew on media containing glucose and antimycin A indicating a low, but significant, level of ADH activity. Plus and minus signs in this column reflect relative growth on YEPD plates containing antimycin A compared to growth on YEPD plates without antimycin A.

**Fig. 2** Localization of  $\beta$ -galactosidase in the indens in cells containing *adr1-lacZ* gene fusions. *a, b*, Mutant 6 (Cys 109 → Tyr); *c, d*, mutant 7 (Cys 106 → Tyr). *a, c*, Immunofluorescence; *b* and *d*, DAPI (4'-6-diamidino-2-phenylindole) staining. These results indicate that the *Adr1*<sup>-</sup> mutants are not defective in nuclear targeting or retention and suggest that they contain full-length fusion proteins. Western analysis of the mutant fusion proteins was consistent with this interpretation (data not shown). The wild-type *ADR1*- $\beta$ -gal fusion protein had previously been shown to be nuclear by both indirect immunofluorescence and by subcellular fractionation whereas an *ADHII*- $\beta$ -gal fusion protein was shown to be cytosolic (manuscript in preparation).

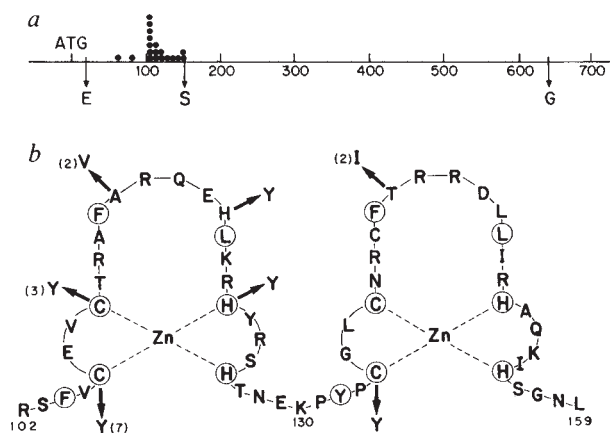
**Methods.** Strain 521-6- $\Delta 1$  carrying YCp642adr1-lacZ plasmids was grown in yeast minimal media containing 5% glucose which was supplemented with an amino-acid mixture lacking tryptophan. Cell fixation and indirect immunofluorescence was performed as previously described<sup>23</sup>. Antiserum against *E. coli*  $\beta$ -gal raised in rabbits (Cappel) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Miles) were used to detect *ADR1*- $\beta$ -gal fusion proteins in cells containing *adr1-lacZ* gene fusions. The percentage of cells which stained was a function of plasmid loss, antibody concentration used and extent of spheroplast formation. Approximately 40–70% of the cells carrying YCp642adr1-lacZ plasmids had an immunofluorescent signal under the condition used; ~60% of the cells carrying the wild-type YCp642ADR1-lacZ plasmid had an immunofluorescent signal under the same conditions. All immunofluorescent staining was nuclear as judged by coincident staining with the DNA stain DAPI.



carboxy-terminal side of the finger domain in *ADR1*. Phenotypically silent mutations must have occurred in this region of the protein. Recent deletion analysis has indicated that the amino-terminal 172 amino acids of *ADR1* act to stimulate *ADH2* transcription (ref. 6, and M. Tavianini, personal communication). Therefore, the results presented here are consistent with the analysis of truncated *ADR1* proteins.

The structure of the proposed fingers and the locations of the mutations are shown in Fig. 3b. Of 17 finger mutations, 14 were

in the first finger. However, amino-acid changes at equivalent positions in both fingers led to the same phenotype. For example, mutations Cys 106 → Tyr and Cys 134 → Tyr both resulted in an *adr1* null phenotype. Mutations Ala 114 → Val and Thr 142 → Ile both led to low *ADH2* expression during glucose growth and to about 15% of the wild-type level of *ADH2* derepression, suggesting that the two *ADR1* finger domains are functionally equivalent and act coordinately. The data also suggest that all members of the finger protein family must contain two or more



**Fig. 3** Positions of mutations in the proposed ADR1 finger structure. *a*, Region of the ADR1 coding sequence which was mutagenized with hydroxylamine. Mutations (small filled circles) map to a limited region of the ADR1 coding sequence. Although the DNA corresponding to this whole region was not sequenced, plasmid reconstruction experiments indicated that these mutations fully account for the ADR1<sup>-</sup> phenotype. The numbers represent ADR1 amino acids. Restriction sites: E, *Eco*RI; S, *Sph*I and G, *Bgl*II. *b*, Amino-acid sequence of the proposed ADR1 finger structure and the positions of *adr1* finger mutations. This structure lies between amino acids 102 and 159 in the ADR1 polypeptide and is drawn according to the model proposed by Miller *et al.*<sup>2</sup>. Invariant cysteine, histidine and hydrophobic residues are circled. Arrows, positions of the ADR1 amino-acid changes in Table 1. The number of independently isolated mutations at each position is given in parentheses.

contiguous repeat units to bind DNA. A number of DNA-binding or transcriptional activation proteins contain only one cysteine-rich repeat unit which resembles that of the finger proteins<sup>6,10-12</sup>. We predict that these proteins will be found to form a different DNA-binding structure than the finger proteins.

Mutations were identified that affected four different amino-acid positions (Cys 106, Cys 109, His 122 and Cys 134) proposed to be zinc ligands (Fig. 3*b*). Replacement of these invariant cysteine or histidine residues with a tyrosine led to an *adr1* null phenotype, consistent with their proposed function in forming a finger structure (Table 1).

Miller *et al.*<sup>2</sup> have proposed that the fingertips in the finger structure make direct contacts with the DNA based on the large number of DNA-binding residues found in this region of the TFIIIA fingers. Only one of the mutations in the ADR1 fingertips (His 118→Tyr) led to an *adr1* null phenotype. His 118 may make a specific contact with DNA or may act in transcriptional activation. The other fingertip mutations, Ala 114→Val and Thr 142→Ile, have an unexpected phenotype. They have a more severe effect on *ADH2* expression during growth on glucose-containing media than on media containing a non-fermentable carbon source. Ala 114 and Thr 142 may also be involved in DNA binding or transcriptional activation but may be less important than His 118.

Analysis of transcription factor TFIIIA led to a proposed model for a DNA-binding domain different from the helix-turn-helix motif<sup>2</sup>. Both structural and genetic studies demonstrated the importance of the helix-turn-helix structure for DNA binding<sup>1</sup>. Although physical evidence supporting the existence of the finger structure is lacking, our genetic data are consistent with the model and strongly support the proposed role of zinc ligands in forming a DNA-binding finger domain.

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## A *c-myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G<sub>0</sub> to G<sub>1</sub>

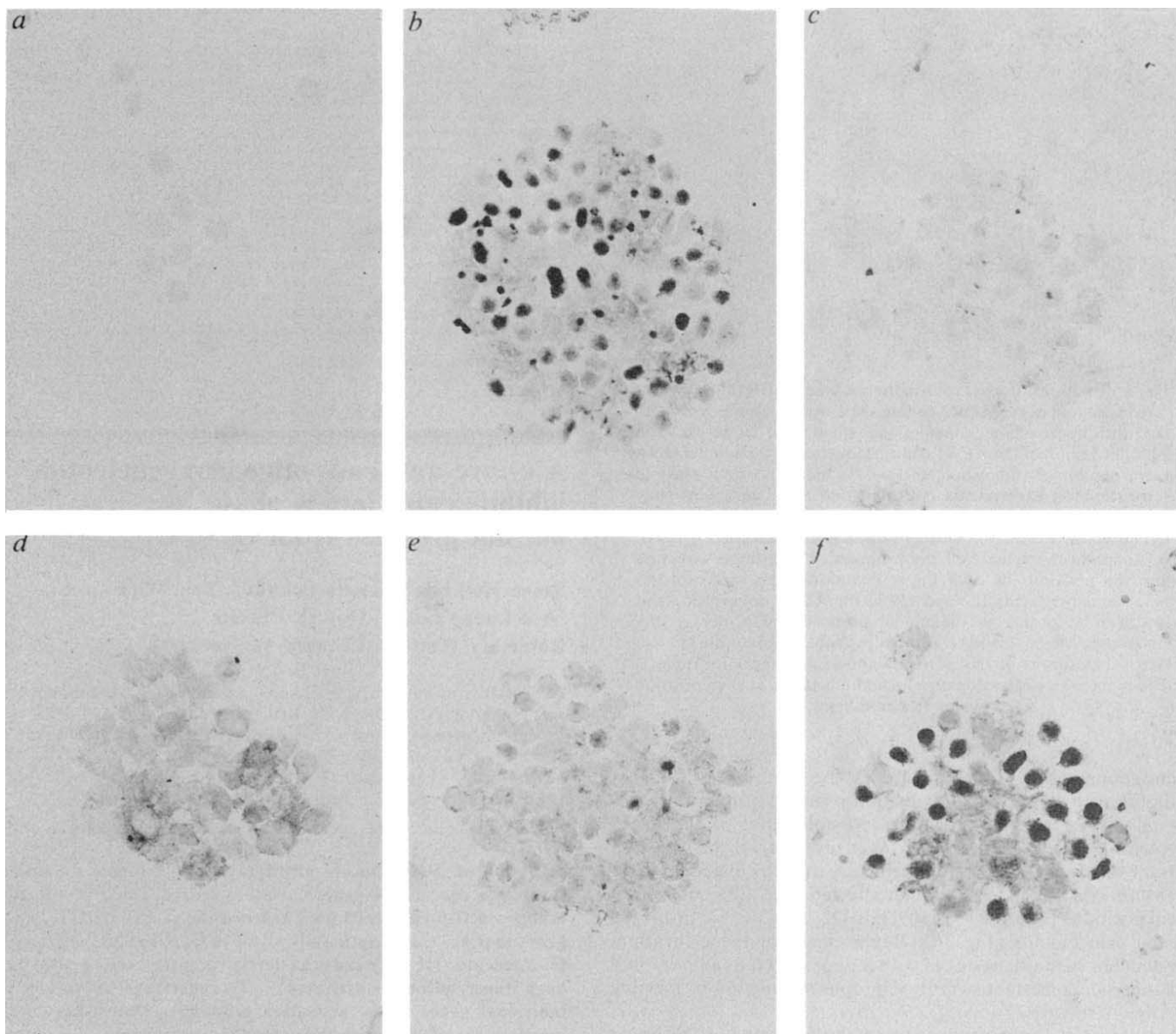
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Initiation of T-lymphocyte proliferation by mitogen or antigen involves a cascade of gene activation events. Thus, by the time mitogen-activated T cells have reached the G<sub>1</sub>/S interface, many genes that are transcriptionally silent in G<sub>0</sub>, like the *c-myc*, IL-2, IL-2 receptor (IL-2R) and transferrin receptor (TfR) genes, have been transcriptionally activated<sup>1-4</sup>. To understand the role of the individual genes in the activation process, one must be able to interfere specifically with the expression or function of each particular gene product. In this way, by blocking the IL-2R with an antibody, it has been demonstrated that IL-2/IL-2R interaction is required to induce TfR expression in activated T cells<sup>3</sup>. When the function or expression of intracellular proteins is to be blocked, however, the need to introduce antibodies into the cytoplasm of viable cells, although possible<sup>5-7</sup>, is a limiting factor. We have taken another approach, namely the exogenous addition to bulk cell cultures of small antisense oligomers. Sequence-specific antisense oligodeoxyribonucleotides have been reported to inhibit intracellular viral replication without interfering with cellular protein synthesis<sup>8,9</sup>. Similarly, rabbit globin mRNA translation in a cell-free system and in rabbit reticulocytes has been inhibited by oligomers complementary to the globin mRNA initiation codon region<sup>10</sup>. Recently, a pentadecadeoxyribonucleotide complementary to the initiation codon and four downstream codons of human *c-myc* mRNA was reported to inhibit the proliferation of the human leukaemic cell line HL-60 specifically<sup>11</sup>. We report here that the same *c-myc* complementary oligonucleotide inhibits mitogen-induced *c-myc* protein expression in human T lymphocytes and prevents S phase entry. Interestingly, *c-myc* antisense treatment did not inhibit G<sub>0</sub> to G<sub>1</sub> traversal as assessed by morphologic blast transformation, transcriptional activation of the IL-2R and TfR genes, or induction of <sup>3</sup>H-uridine incorporation.

The specific role(s) of the *c-myc* gene in lymphocyte mitogenesis is unknown. Initiation of *c-myc* transcription occurs



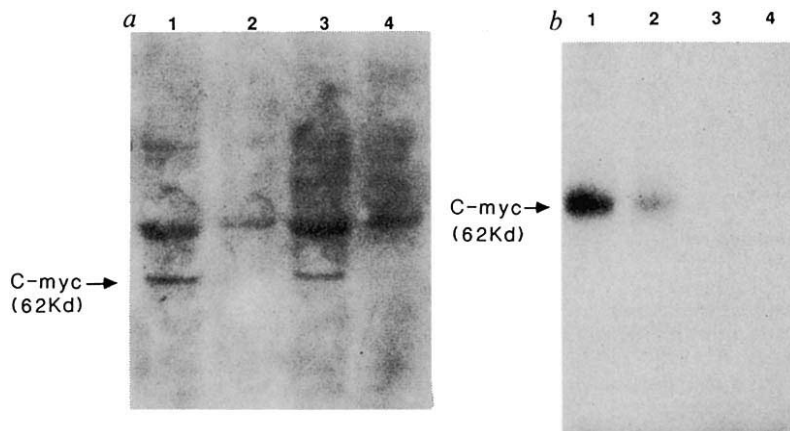


**Fig. 1** Inhibition of PHA-stimulated *c-myc* protein expression in peripheral blood lymphocytes by an antisense oligomer to *c-myc* mRNA. *a*, Unstimulated lymphocytes stained with anti-*c-myc* antibody; *b*, PHA-stimulated cells stained with anti-*c-myc* antibody; *c*, PHA-stimulated cells stained with anti-*c-myc* antibody which had been preincubated with a twofold excess of recombinant *c-myc* protein; *d*, PHA-stimulated cells stained with irrelevant mouse IgG<sub>1</sub>; *e*, *c-myc* antisense-pretreated, PHA-stimulated cells stained with anti-*c-myc* antibody; *f*, VSV M protein antisense-pretreated, PHA-stimulated cells stained with anti-*c-myc* antibody.

**Methods:** Peripheral blood mononuclear cells were isolated from 20 ml venous blood of a normal donor by density centrifugation. Upon isolation, the cells were comprised of 73% T lymphocytes, 10% B lymphocytes and 17% monocytes by flow cytometric analysis<sup>3</sup>. After 10 h of culture, non-adherent cells consisted of 85% T lymphocytes, 10% B lymphocytes and 5% monocytes. Cell viability remained greater than 90% under all conditions (as determined by trypan blue exclusion). Cells were cultured (37 °C, 5% CO<sub>2</sub>, humidified atmosphere) at a concentration of  $1 \times 10^6 \text{ ml}^{-1}$  ( $10^5$  per well) in a 96-well U-bottom microtitre plate (Costar) in RPMI 1640 containing 10% fetal bovine serum and antibiotics (BRL). Some wells were pretreated with 30  $\mu\text{M}$  antisense oligomer to either human *c-myc* mRNA or VSV M protein mRNA. Oligomer sequences were complementary to the first 5 codons (starting from the initiation codon) of human *c-myc* mRNA<sup>11</sup> and nucleotides 17–31 of VSV M protein mRNA<sup>18,23</sup>. Oligomer synthesis was performed as described elsewhere<sup>24</sup> and oligomers were purified by reverse-phase high-pressure liquid chromatography on a C<sub>18</sub> column eluted with a 0–25% acetonitrile gradient in 50 mM triethyl-ammonium acetate (pH 6.0). After a 4-h incubation with or without oligomers, PHA (2  $\mu\text{g ml}^{-1}$ ) was added to all wells except the unstimulated controls. Six hours later, cells were removed and centrifuged onto microscope slides with a cytocentrifuge (Shandon, Inc.). After air drying for ~30 min, the slides were fixed for 30 min in freshly prepared paraformaldehyde (1% in Tris-buffered saline). After 3 washes in Tris-buffered saline (containing 1% normal goat serum), slides were stained with one of the following: 1, murine monoclonal anti-human *c-myc* antibody<sup>19</sup> (1:500 dilution of ascites); 2, an irrelevant mouse ascites preparation of the same immunoglobulin class and concentration; 3, murine anti-*c-myc* antibody which had been preincubated with a twofold excess of purified recombinant human *c-myc* protein<sup>21</sup>. The staining procedure was that of Hsu *et al.*<sup>25</sup>, and was performed immediately after fixation as delayed staining resulted in a loss of nuclear localization and appearance of cytoplasmic anti-*c-myc* reactivity.

**Fig. 2.** Inhibition of *c-myc* protein accumulation following PHA-stimulation of peripheral blood lymphocytes by an antisense oligomer of *c-myc*. **a**, Western blot analysis. Lane 1, PHA; lane 2, unstimulated; lane 3, *myc* nonsense pretreated/PHA treated; lane 4, *myc* antisense pretreated/PHA treated. **b**, Immunoprecipitation of metabolically labelled cell lysates. Lane 1, PHA; lane 2, *myc* sense pretreated/PHA treated; lane 3, *myc* antisense pretreated/PHA treated; lane 4, unstimulated.

**Methods.** **a**, Following treatment conditions as described in Fig. 1,  $5 \times 10^6$  cells were washed twice in cold PBS, solubilized in sample loading buffer (containing 2-mercaptoethanol), electrophoresed on an 8% polyacrylamide gel, transferred to nitrocellulose and reacted with a murine monoclonal anti-*myc* antibody (3C7) as described by Evan *et al.*<sup>19</sup>. The goat anti-mouse second antibody nonspecifically reacted with another band larger than *c-myc*, which could be reduced in intensity by extensive washing of the membrane. This other band does not appear if metabolically labelled samples are immunoprecipitated. **b**, Cells were labelled with 1 mCi each of <sup>35</sup>S-methionine and <sup>35</sup>S-cysteine during the time the treatments were administered as in Fig. 1 (10 h). After treatment,  $5 \times 10^6$  cells in each group were lysed in 0.01 M Tris-Cl (pH 7.5), 0.144 M NaCl, 0.5% NP-40, 0.5% SDS, 0.1% Trasylol, 1 mM phenylmethylsulphonyl fluoride and 10 mM iodoacetamide. After 30 min on ice (with vortexing every 10 min) the lysates were centrifuged at 10,000g for 10 min. The lysates were then adjusted to 1 ml and incubated for 1 h with 0.1 ml of protein A-coated Sepharose beads which had been pretreated with normal mouse serum. After centrifugation, the lysates were incubated overnight with protein-A coated Sepharose beads to which excess 3C7 antibody had been adsorbed. After 5 washes in lysis buffer to which 0.5% deoxycholate had been added, the bead pellets were resuspended in 4× sample loading buffer (containing 2-mercaptoethanol), boiled, electrophoresed and analysed as described by Hann *et al.*<sup>20</sup>.



within a few hours of mitogen addition to T lymphocytes and has been suggested to be important in the  $G_0$  to  $G_1$  transition<sup>12,13</sup>. Interestingly, antibodies directed against the *c-myc* protein inhibit DNA polymerase activity in nuclei isolated from human cells<sup>14</sup>. But accumulation of *c-myc* transcripts is not sufficient to support T-lymphocyte proliferation<sup>15-17</sup>. Whether *c-myc* induction is required for proliferation is not known. We have addressed this question by attempting to inhibit *c-myc* protein expression with antisense oligomers. Freshly isolated peripheral blood lymphocytes, considered to be  $G_0$  cells, were used in these experiments. An oligodeoxyribonucleotide complementary to the first five codons of human *c-myc* messenger RNA (5'-dAACGTTGAGGGGCAT-3'), which were predicted to form a large hairpin loop<sup>11</sup>, was added to cells at a final concentration of 30  $\mu$ M 4 hours before addition of the mitogenic lectin, phytohaemagglutinin (PHA-P, Sigma). As a control, the same concentration of a pentadecadeoxyribonucleotide complementary to nucleotides 17-31 of vesicular stomatitis virus (VSV) M protein mRNA was used (5'-dTGGGATAACACTTA-3'; ref. 18). In addition, oligomers termed 'sense' and 'nonsense' were studied. The *c-myc* sense oligomer was complementary to the antisense construct, while the nonsense oligomer contained the same bases as the antisense construct, but these were randomly scrambled (5'-dCTGAAGTGGCATGAG-3'). Intracellular *c-myc* protein was detected in cytospin preparations ( $10^5$  cells per slide) using a murine monoclonal anti-human *c-myc* antibody (provided by Dr G. Evan<sup>19</sup>). *C-myc* protein was also quantified by Western blot analysis as described by Evan *et al.*<sup>19</sup>, and by immunoprecipitation of <sup>35</sup>S-cysteine and <sup>35</sup>S-methionine metabolically-labelled cell lysates as described by Hann *et al.*<sup>20</sup>. Resting ( $G_0$ ) T cells possessed no detectable *c-myc* protein reactivity (Fig. 1a; Fig. 2a, lane 2). However, within 6 h of PHA addition, 30-40% of the cells demonstrated strong nuclear reactivity with the *c-myc* antibody (Fig. 1b; Fig. 2a, lane 1). Prior incubation of the antibody with recombinant *c-myc* protein<sup>21</sup> completely blocked the staining (Fig. 1c). Staining with a non-reactive mouse ascites of the same isotype and the same immunoglobulin concentration also produced no reactivity (Fig. 1d). Preincubation of the cells for 4 h with 30  $\mu$ M *c-myc* antisense, markedly reduced the number (to 10%) and intensity of *c-myc* protein positive cells 6 h after PHA addition (Fig. 1e; Fig. 2a, lane 4). Antisense concentrations lower than 30  $\mu$ M were less inhibitory, although doses as low as 15  $\mu$ M produced

some diminution in *c-myc* expression (data not shown). Pre-treatment with *c-myc* antisense markedly inhibited the synthesis of *c-myc* protein as detected by immunoprecipitation of metabolically labelled material (Fig. 2b). Preincubation with VSV M protein antisense, *c-myc* sense, or *c-myc* nonsense oligomers had little inhibitory effect on *c-myc* expression (Fig. 1f; Fig. 2a, lane 3; Fig. 2b, lane 2).

At 20 h after mitogen stimulation, the *c-myc* protein level of control lymphocytes was lower than at 6 h, but it was still inhibited in the *c-myc* antisense pretreated cells (data not shown). Thus, consistent with the findings of Persson *et al.*<sup>4</sup>, *c-myc* protein levels follow a time course similar to that previously reported for *c-myc* gene transcription<sup>1,12</sup>, and one addition of antisense oligomer is sufficient to inhibit appearance of the *c-myc* protein substantially throughout this time course. But if PHA is added 24 or 48 h after oligomer pretreatment, *c-myc* protein can be induced to the same degree as in the PHA control samples (data not shown). These results, taken together with viability studies (by trypan blue exclusion) made at 72 h after PHA addition, suggest that oligomer addition itself is not toxic and that the oligomer is sufficiently degraded within about 24 h to be ineffective.

Using *c-myc* antisense, we then examined the effects of inhibition of *c-myc* protein expression on PHA-stimulated cell proliferation. Initial attempts to study proliferative capacity using <sup>3</sup>H-thymidine proved impossible as both oligomers reduced <sup>3</sup>H-thymidine incorporation into cellular DNA by >80% (cells were pulsed at +72 h for 4 h with 1  $\mu$ Ci <sup>3</sup>H-thymidine). Synthetic oligomers have previously been reported to reduce <sup>3</sup>H-thymidine incorporation into cellular DNA nonspecifically<sup>8</sup>. Eighteen hours after addition of d-(Tp)<sub>8</sub>[<sup>3</sup>H]T, a tritiated dT homo-octamer, to mammalian cells, only 70% of the label remained associated with the oligomer, whereas the other 30% was found associated with thymidine triphosphate and cellular DNA, indicating the occurrence of intracellular oligomer degradation with subsequent reuse of the thymine base<sup>22</sup>. When a d-(Ap)<sub>8</sub>A (dA homo-octamer) analogue was tested, it had no effect on <sup>3</sup>H-thymidine incorporation assays<sup>22</sup>. As the oligomer complementary to *c-myc* contains three T's and the oligomer complementary to VSV M protein contains five T's, these reagents could significantly increase the intracellular thymidine pool at +72 h, thereby greatly diluting the specific activity of this pool after pulse-labelling with 1  $\mu$ Ci exogenous thymidine. To cir-



**Table 1** Effect of an antisense oligodeoxyribonucleotide complementary to *c-myc* mRNA on mitogen-stimulated lymphocyte proliferation

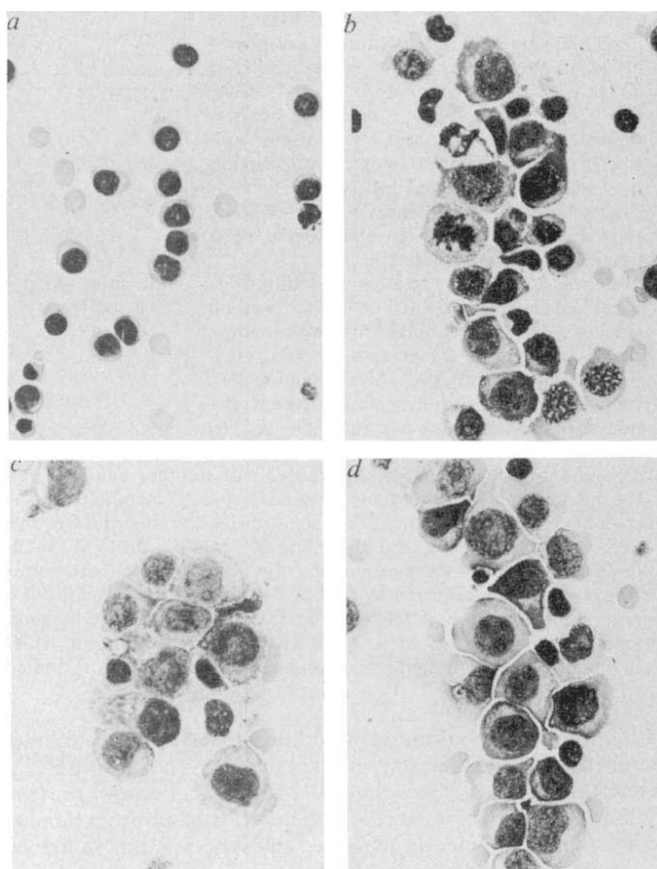
|                                                                            | G <sub>0</sub> /G <sub>1</sub><br>(%) | S<br>(%)       | G <sub>2</sub> /M<br>(%) | Mitotic<br>index<br>(%) |
|----------------------------------------------------------------------------|---------------------------------------|----------------|--------------------------|-------------------------|
| Untreated cells                                                            | 78<br>(78, 78)                        | 8<br>(7, 9)    | 14<br>(13, 15)           | <1                      |
| PHA-stimulated cells                                                       | 53<br>(52, 54)                        | 24<br>(20, 27) | 24<br>(21, 26)           | 5                       |
| PHA-stimulated cells<br>Pretreated with antisense<br>to VSV M protein mRNA | 50<br>(50, 50)                        | 30<br>(30, 30) | 20<br>(20, 20)           | 4                       |
| PHA-stimulated cells<br>pretreated with antisense<br>to <i>c-myc</i> mRNA  | 78<br>(73, 82)                        | 7<br>(3, 10)   | 16<br>(15, 17)           | <1                      |

Freshly isolated normal peripheral blood lymphocytes were cultured as described in the legend to Fig. 1. Some wells were pretreated for 4 h with 30  $\mu$ M oligomer complementary to either *c-myc* or VSV M protein mRNA. All wells except the unstimulated controls then received 2  $\mu$ g ml<sup>-1</sup> PHA. Three days later, cells were removed and DNA cell-cycle phase analysis was performed according to the method of Braylan *et al.*<sup>26</sup>. Essentially, cells were washed in phosphate-buffered saline, fixed for 1 h in 50% ethanol at 4 °C, incubated with 500 units ml<sup>-1</sup> RNase A (Worthington; boiled to destroy contaminating DNase activity) for 30 min at 37 °C, and incubated for 30 min at room temperature with propidium iodide (25  $\mu$ g ml<sup>-1</sup>, Calbiochem). Samples were analysed on a FACS II flow cytometer (Becton-Dickinson FACS Systems) using the 488-nm line of an argon laser for excitation. Quantification of cell-cycle phase distributions was performed on an integrated PDP 11/34 computer (DEC) using software developed by the Division of Computer Research and Training, NIH<sup>27</sup>. We have routinely observed that 75–85% of freshly isolated peripheral blood lymphocytes are in G<sub>0</sub>/G<sub>1</sub>, whereas 45–55% of continuously proliferating cells are in G<sub>0</sub>/G<sub>1</sub> at any given time. The cell-cycle data are expressed as the mean of two separate experiments with the individual values in parentheses. Mitotic indices were determined on 72-h PHA-stimulated samples with or without antisense oligomers. Cells were centrifuged onto glass slides and stained with Wright's stain. The number of mitoses per 1,000 cells was then determined microscopically. The mitotic index for PHA-activated lymphocytes typically averages about 5% (ref. 28).

cumvent this difficulty, we assessed proliferation using two independent techniques—DNA cell-cycle analysis and determination of mitotic index.

DNA histograms were generated at 72 h after PHA addition (Table 1). Cells were fixed, reacted with the DNA intercalating dye propidium iodide and analysed by flow cytometry. Of the untreated cells, 78% remained in G<sub>0</sub>/G<sub>1</sub> at this time, whereas only 53% of the stimulated cells did so. Although pretreatment with VSV M protein antisense had no effect on the proliferation of PHA-treated cells (50% in G<sub>0</sub>/G<sub>1</sub>), pretreatment with *c-myc* antisense resulted in 78% of the cells remaining in G<sub>0</sub>/G<sub>1</sub> 72 hours after mitogen addition. These results were confirmed by quantifying the mitotic indices of cell populations 72 h after PHA stimulation in the presence or absence of antisense oligomers (Table 1). The mitotic index is another measure of a cell population's proliferative capacity because mitoses can be observed only in cells which have attained a tetraploid DNA content before actual cell division. Mitotic indices were determined from Wright's stained-cell preparations similar to those pictured in Fig. 3 and were found to be reduced by more than 80% in *c-myc* antisense pretreated cultures (comparable to the frequency seen in unstimulated cells). VSV M protein antisense pretreatment did not affect the mitotic index. *C-myc* antisense pretreatment thus inhibited PHA-stimulated DNA synthesis, indicating that *c-myc* protein expression is required for this to occur.

We next studied the effects of inhibition of *c-myc* protein expression on G<sub>0</sub> to G<sub>1</sub> traversal by following the morphological changes induced by PHA in the presence and absence of *c-myc*



**Fig. 3** PHA-stimulated blastogenesis after *c-myc* antisense pretreatment. Peripheral blood lymphocytes were isolated and cultured as described in the legend to Fig. 1 and Table 1. Three days after PHA treatment, cells were centrifuged onto glass slides and stained with Wright's stain to reveal basic cellular morphology. Pretreatment with antisense oligomer to either *c-myc* (c) or VSV M protein (d) did not prevent PHA-stimulated blastogenesis (b) when compared to unstimulated controls cultured for 3 days (a).

antisense pretreatment; the expression of the proliferation-associated genes for IL-2R and TfR, which are known to be activated subsequent to *c-myc*<sup>1</sup>; and induction of <sup>3</sup>H-uridine incorporation. Expression of IL-2R and TfR was examined 48 h after PHA addition. Prior exposure to *c-myc* antisense did not block the mitogen-stimulated appearance of these receptors on the cell surface (Table 2). As TfR is known to be expressed after IL-2R expression in late G<sub>1</sub> (refs 1, 3, 16), our data indicate that even when *c-myc* protein expression is substantially reduced, the cells are able to traverse from G<sub>0</sub> to G<sub>1</sub> and into late G<sub>1</sub>. It has been shown previously that IL-2R may be induced in the absence of *c-myc* induction<sup>2</sup>, consistent with the fact that IL-2R gene transcriptional activation is not dependent on protein synthesis<sup>1</sup>. Our results suggest that TfR expression is also independent of *c-myc* expression, although we cannot rule out a dependence on the very low levels of *c-myc* protein observed in *c-myc* antisense-treated cells. On the other hand, IL-2R and TfR expression are not sufficient to support DNA synthesis in the absence of normally induced amounts of the *c-myc* protein.

Uridine incorporation was monitored 18–24 and 24–48 h after PHA stimulation (Table 2). *C-myc* antisense pretreatment failed to block PHA-stimulated induction of <sup>3</sup>H-uridine incorporation measured at either time point, supporting our hypothesis that *c-myc* antisense-pretreated cells are capable of entering G<sub>1</sub> upon PHA stimulation. In accordance with this observation, the morphological alterations characteristic of lymphocyte

**Table 2** Effects of an antisense oligodeoxyribonucleotide complementary to *c-myc* mRNA on mitogen-stimulated IL-2 receptor expression, transferrin receptor expression and <sup>3</sup>H-uridine uptake

|                                                                             | % Positive cells<br>IL-2<br>receptor | % Positive cells<br>Transferrin<br>receptor | <sup>3</sup> H-uridine<br>incorporation (c.p.m.) |
|-----------------------------------------------------------------------------|--------------------------------------|---------------------------------------------|--------------------------------------------------|
| Unstimulated cells                                                          | 8<br>(8, 8)                          | 1<br>(0, 1)                                 | 506<br>(424-911)                                 |
| PHA-stimulated cells                                                        | 40<br>(32, 47)                       | 30<br>(23, 37)                              | 1091<br>(898-2726)                               |
| PHA-stimulated cells<br>pretreated with anti-<br>sense to <i>c-myc</i> mRNA | 33<br>(30, 35)                       | 21<br>(16, 26)                              | 1454<br>(953-1470)                               |
| PHA-stimulated cells<br>pretreated with anti-<br>sense to VSV M Protein     | 37<br>(31, 42)                       | 26<br>(23, 29)                              | 1197<br>(1011-1324)                              |

Peripheral blood lymphocytes were cultured as described in legend to Fig. 1. Two days after PHA treatment ( $2 \mu\text{g ml}^{-1}$ ) cells were analysed for surface expression of IL-2 receptors and transferrin receptors using the monoclonal antibodies anti-Tac (a gift of Dr T. Waldmann) and OKT9 (Ortho Immunology Systems) respectively. Analyses were performed using a FACS II flow cytometer as described elsewhere<sup>3</sup>. Data are expressed as the mean percent positive cells from two experiments with the individual values in parentheses. Uridine incorporation was determined by labelling cells with  $1 \mu\text{Ci ml}^{-1}$  <sup>3</sup>H-uridine (Dupont/NEN,  $38.9 \text{ Ci mmol}^{-1}$ ) within 18–24 h of PHA treatment. Cells were harvested by precipitation with trichloroacetic acid and filtration through Whatman GFC filter disks. Acid precipitable radioactivity was determined by liquid scintillation counting. Data are expressed as the median value of three determinations with the range in parentheses. Uridine incorporation was also determined within 24–48 h of PHA addition. Acid-precipitable radioactivity was then increased 7.8-fold above control in the PHA group, 5.6-fold in the *c-myc* antisense group and 4.5-fold in the VSV antisense group.

mitogenesis were not blocked by *c-myc* antisense pretreatment (Fig. 3). The cells underwent a marked increase in size and decrease in nuclear to cytoplasmic ratio following PHA treatment. Furthermore, the metabolic rate of *c-myc* antisense pretreated cells (as measured by pH of the culture medium) was as great as that of the PHA controls (data not shown). These results do not support a role for *c-myc* in the  $G_0$  to  $G_1$  transition process of normal T lymphocytes, unless the greatly diminished level of *c-myc* protein detected in the *c-myc* antisense treated cells is sufficient. Rather, the *c-myc* gene product would appear to be critical for S phase entry and/or S phase traversal. This would be consistent with the recently suggested involvement of the *c-myc* protein in DNA synthesis<sup>14</sup>.

We have shown that antisense oligonucleotides can be used to inhibit the expression of the *c-myc* protein in mitogen-stimulated lymphocytes, allowing study of the role of *c-myc* gene expression in lymphocyte mitogenesis. Furthermore, the use of sequence-specific antisense oligomers may allow study of the functions of other intracellular proteins.

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## Kinetics of flight muscles from insects with different wingbeat frequencies

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Small insects usually beat their wings at a higher frequency than large insects. For energetic reasons, the wingbeat frequency is close to the natural resonant frequency of the wing movement<sup>1–3</sup>. In the flight muscles of many insects, termed fibrillar muscles, the contractions are myogenic: the muscle is only partially activated by calcium; full activation requires mechanical stretch. Demembrated fibres can be stretch-activated *in vitro*, allowing both tension changes and the ATPase activity of the fibres to be monitored. The tension response to a sudden length change shows a large delayed tension component (Fig. 1), whose rate-constant determines the frequency at which the muscle can deliver maximum power to the wings. We show here that this rate-constant is roughly proportional to wingbeat frequency in the flying insect. We find that the ATPase rate is slower and is not related to the rate-constant of tension production, and show that insects with widely different wingbeat frequencies have muscles with similar mechanical power output. We conclude: (1) that the rate-limiting step in the cross-bridge cycle, which limits the ATPase activity, follows the state in which maximum active tension is produced and does not limit the rate at which tension is generated; (2) that the first tension-generating state is probably an actomyosin.ADP.Pi state; and (3) that a preceding step, probably an actomyosin.ADP.Pi isomerization, which controls the rate-constant for force generation, is faster in smaller insects than in larger ones.

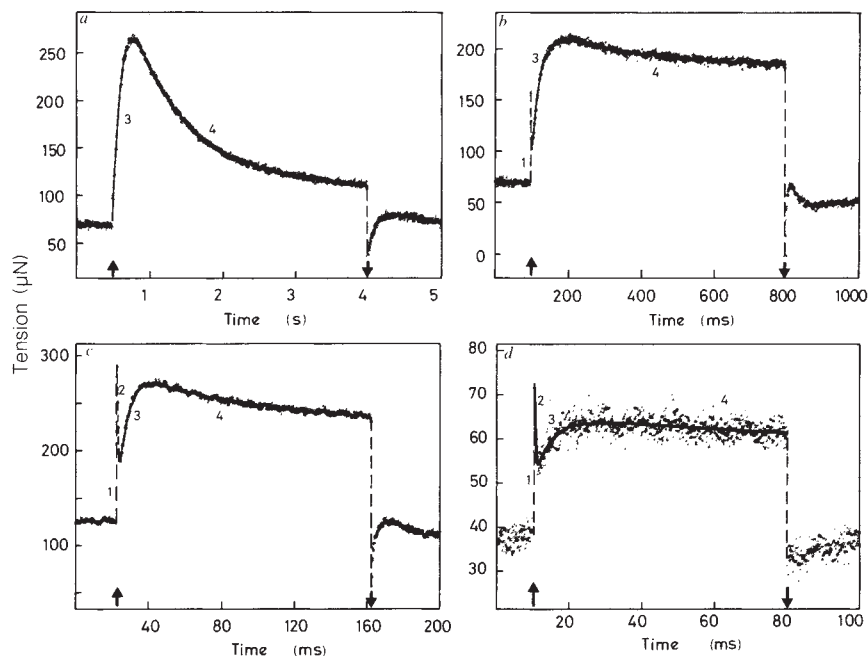
We recorded the wingbeat frequencies of different insect species either by tape-recording the sound of the flying insect and then replaying the tape through an oscilloscope; or by mounting the insect on a gramophone pickup and recording on an oscilloscope; or by both methods. We were not able to induce flight in the waterbug *Lethocerus colossicus*; the wingbeat frequencies of species of similar size have been measured previously<sup>4</sup>. The ambient temperature was about 25 °C.

We performed mechanical and biochemical experiments on single demembrated muscle fibres that had been dissected from the insect thorax and mounted on a mechanical testing

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**Fig. 1** Transient tension response of muscle from four species of insect to applied length changes. Short lengths (0.5–2 mm) of glycerol-extracted muscle fibre from: *a*, the giant waterbug *L. indicus*; *b*, the crane fly *Tipula oleracea*; *c*, the hoverfly *Syrphus ribesii*; and *d*, the fruitfly *D. melanogaster* were attached to T-clips<sup>24</sup> and mounted on a mechanical apparatus<sup>6</sup> but with a faster motor<sup>5</sup>. Diameters of preparations, 60–80  $\mu\text{m}$ . Experimental procedure as ref. 6. Fibres activated by immersion in activating solution containing 10 mM  $\text{Na}_2\text{ATP}$ , 10 mM  $\text{MgCl}_2$ , 10 mM creatine phosphate, 2 mg  $\text{ml}^{-1}$  creatine kinase, 5 mM CaEGTA, 20 mM histidine, pH 7.0, 15 °C. Length of the muscle was increased rapidly by  $\sim 1\%$  at the first arrow, held at the increased length for a short time as indicated and then returned to the original length at the second arrow. The tension response to the rapid length increase shows four phases (1–4). Phase 3 is the large delayed-tension component whose rate is faster in insects with higher wingbeat frequencies. The tension responses of phases 2, 3 and 4 were fitted by sums of three exponential processes using the program DISCRETE<sup>7</sup>. The fitted curves are shown for phases 3 and 4 (and for phase 2 in *c* and *d*). Dots representing the recordings result from sampling by the digital oscilloscope. Phases 1 and 2 are too fast to be seen on the displayed timebase in *a* and *b*.



apparatus<sup>5,6</sup>. We activated the fibres by immersion in a solution containing MgATP and free  $\text{Ca}^{2+}$  (Fig. 1 legend) and measured the transient tension responses to a step change in length for each species (Fig. 1). This is the first time that rapid kinetic measurements have been recorded on muscles as small as those from the fruitfly *Drosophila melanogaster* (1 mm long).

We analysed the time course of the response as a sum of exponentials<sup>7</sup>, and plotted the rate-constant of the 'delayed-tension' phase ( $r_3$ ) against wingbeat frequency ( $f_{\text{WB}}$ ) (Fig. 2a). The relationship between the rate-constant for tension production and wingbeat frequency is given by:

$$r_3 = 1.3 \times f_{\text{WB}} + \text{constant} \quad (1)$$

Under forced sinusoidal oscillation, the activated fibres deliver work into the driving apparatus<sup>8</sup>. We measured the frequency of maximum power output ( $f_{\text{PMAX}}$ ) under forced length oscillations of 3% peak-to-peak amplitude at 15 °C, and find that it is related to  $r_3$ :

$$r_3 = 6.0 \times f_{\text{PMAX}}^{15^\circ\text{C}} \quad (2)$$

Therefore,

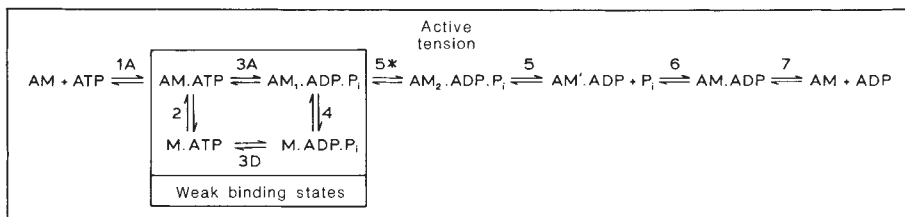
$$f_{\text{PMAX}}^{15^\circ\text{C}} = 0.22 \times f_{\text{WB}}^{\text{Thoracic temp.}} + \text{constant} \quad (3)$$

For all the species tested the maximum power output occurred at a lower frequency than the wingbeat frequency in the intact insect and with a lower power output<sup>9</sup>. The main explanation for this is temperature. We made the mechanical measurements on the single, skinned fibres at a low temperature (15 °C) to minimize problems of nucleotide diffusion across the fibres, but measured the wingbeat frequencies at an ambient temperature of 25 °C. Further, the thoracic temperature in many insects,

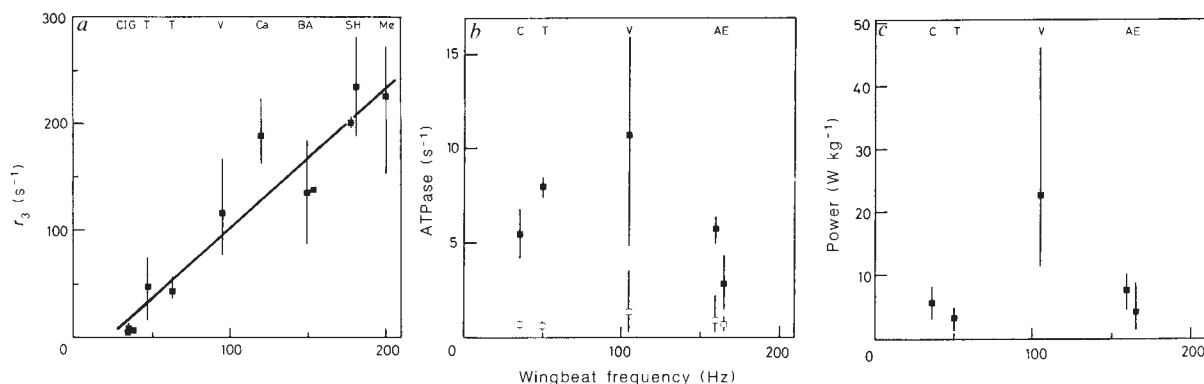
particularly large ones, is known to be raised during flight. The thoracic temperature of flying waterbugs is 40 °C (*L. indicus* and *L. griseus*; J.E.M., J. Lund and V.K., unpublished data); of the bees *Apis* and *Bombus* is 39 and 40 °C, respectively<sup>10,11</sup>; whereas for the small glabrous insects we find values of 27 °C in *Tipula* and 29 °C in *Vespa*. If  $r_3$  were measured at the flight temperature, the effect would be to reduce the intercept of Fig. 2a by raising the points for larger insects with lower wingbeat frequencies more than for the smaller insects with higher wingbeat frequencies. The constant of proportionality (0.22) in equation (3) will also be much closer to unity if the temperature difference between the mechanical experiments and flight is taken into account.

One other factor that could affect the frequency of maximum power output is the amplitude of the length oscillation. The amplitude of length changes of the muscle during flight is not known accurately for any insect, and may well be different in different species. We used a constant amplitude of 3% for consistency.

We measured the fibre ATPase activities of five species by determining the rate of production of ADP in the incubation baths, measuring the ADP concentrations by HPLC<sup>12</sup>. We determined ATPase activities in relaxed and in activated muscle under oscillating conditions in which the fibres were developing their maximum power output (Fig. 2b, c). ATPase activities were independent of the diameter of the fibre preparation (up to 80  $\mu\text{m}$ ) or the concentration of MgATP used above 10 mM [MgATP], indicating that diffusion of ATP into the fibres is not rate-limiting. The ATPase rate and mechanical power output in the intact insects would be considerably greater for those insects that show an elevated thoracic temperature during flight. The ATPase activity of activated *Lethocerus* fibres increases by a



Scheme 1: The crossbridge cycle. See opposite.



**Fig. 2** *a*, Relationship between wingbeat frequency and rate constant of the delayed-tension component (phase 3),  $r_3$ , of the transient response to a step length change for different insects. *b*, ATPase activities of fibres measured in relaxing ( $\square$ ) and in activating ( $\blacksquare$ ) solution with the fibres oscillated sinusoidally with a peak-to-peak amplitude of 3% at the frequency giving the maximum mechanical power output. *c*, Power output against wingbeat frequency, measured from the area of length-tension diagrams obtained during 3% sinusoidal length oscillation of the fibres at the frequency giving maximum power output. *a*, Rate constants determined as described in Fig. 1. The mean value of  $r_3$  and the total spread of values obtained are shown. The straight line through the data is the least-squares fit. With the exception of the specimens of the giant waterbug (genus *Lethocerus*) the insects were local: *L. colossicus* (Jamaica), *L. indicus* (Thailand) and *L. griseus* (Florida). Letters indicate the species of insect: C, *L. colossicus*; I, *L. indicus*; G, *L. griseus* (giant waterbugs); T, *Tipula* spp. (crane flies); V, *Vespa vulgaris* (wasp); Ca, *Calliphora erythrocephala* (blowfly); B, *Bombus terrestris* (bumblebee); A, *Apis mellifera* (honeybee); S, *Syrphus ribesii* (hoverfly); E, *Episyrphus balteatus* (hoverfly); H, *D. hydei*; Me, *D. melanogaster* (fruitflies). *b*, Activating solution contained 18.5 mM  $\text{Na}_2\text{ATP}$ , 15.5 mM  $\text{MgCl}_2$ , 6 mM CaEGTA, 20 mM histidine and inhibitors for other ATPase activity (1 mM  $\text{NaN}_3$ , 10  $\mu\text{M}$  diadenosine pentaphosphate, 0.5 mM quercetin and 1  $\mu\text{g ml}^{-1}$  oligomycin) at pH 7.0. Relaxing solution was identical, but with 5 mM EGTA in place of the 6 mM CaEGTA. Temperature 20 °C. The myosin active site (S1) concentration in each fibre was estimated by densitometry of the myosin heavy-chain band in calibrated SDS polyacrylamide gels. *c*, Values of power output: *L. colossicus*, 5 Hz; *Tipula*, 17 Hz; *Vespa*, 27 Hz; *Apis*, 35 Hz; *Episyrphus*, 75 Hz. Solutions and conditions as for *b*. Power outputs measured at 20 °C. The wingbeat frequencies in the intact flying insects were determined at an ambient temperature of 25 °C; note that the thoracic temperatures during flight are above ambient (see text).

factor of about 2.3 for a 10 °C rise in temperature<sup>13</sup>. It is clear from these results that the rate of ATP hydrolysis is not correlated with, and is always slower than, the rate-constant for delayed tension, and that with the exception of *Vespa* we find no significant change in fibre ATPase activity with wingbeat frequency.

We can draw two main conclusions from our results: (1) the wide variation in rate-constants observed for the development of the delayed tension, together with the lower and relatively constant values for the ATPase activity of the fully working fibres, shows that the main tension-generating state of the cross-bridge cycle precedes the rate-limiting step of the cycle; and (2) the relatively constant power output and ATPase activity in most species requires the work per oscillatory cycle in the flying insect to be generally smaller in insects with higher wingbeat frequencies.

Current work with vertebrate striated muscle shows disagreement about which step of the crossbridge cycle is rate-limiting and which is the main tension-generating state<sup>14-17</sup>. Phosphate-water-oxygen exchange experiments with maximally active, skinned *Lethocerus* flight muscle fibres<sup>12</sup> show that the Pi-release process (the steps following ATP cleavage and up to Pi release) is rate-limiting for their ATPase cycle. That work, together with this, implies that a state preceding Pi-release is the first tension-generating state in the crossbridge cycle. In vertebrate muscle the first attached state following ATP hydrolysis is a weak-binding actomyosin-ADP-Pi state (AM-ADP-Pi) in which no tension is developed, and which is in rapid equilibrium with the corresponding detached state<sup>17,18</sup>. If this is also the case for insect flight muscle, then the first tension-generating process must be an isomerization (step 5\* in scheme 1) between two AM-ADP-Pi states. The full scheme for the crossbridge cycle is then as shown in scheme 1<sup>19</sup>.

The rate of oxygen consumption has been used for many previous estimates of the power output of flying insects. The only systematic study of insects of different size that we know of<sup>20</sup>, that of the oxygen consumption in hovering euglossine bees, shows that the mass-specific rate of oxygen consumption is much greater in smaller bees. In this restricted group it is

possible that the mechanical power output does vary with wingbeat frequency. A recent theoretical study of power output<sup>21</sup>, based on the energy available from mitochondria, also suggests that insects with fibrillar muscle should have higher mass-specific power output with higher wingbeat frequencies, requiring the work per cycle to be independent of size. This is contrary to our findings for most species.

The rate of oxygen consumption by unrelated species of insect has been measured many times<sup>22,23</sup>, but no clear relationship between power input and insect size has emerged. Our results agree with the conclusion that mass-specific oxygen consumption does not scale with either mass or wingbeat frequency for most insects.

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# Differential polarization microscopy of changes in structure in spermatocyte nuclei

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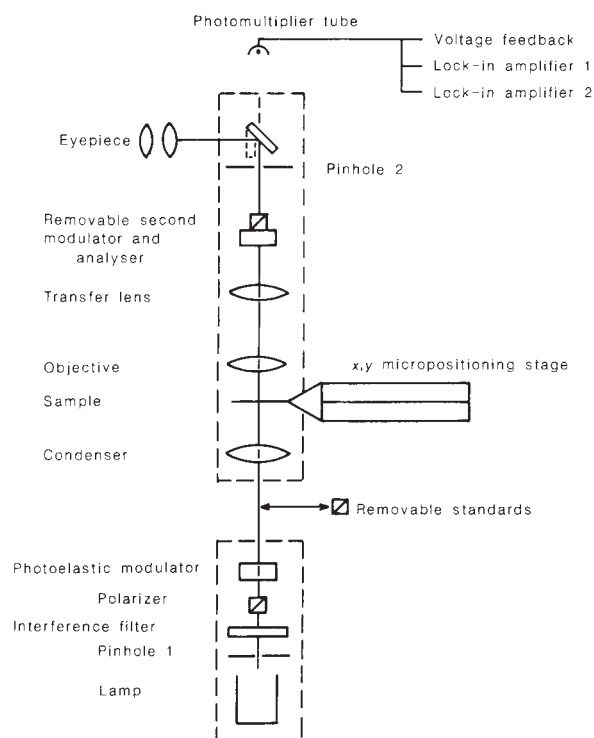
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Phase-dependent forms of microscopy (such as phase contrast, interference, or polarization microscopy) have been used for many years; the amount of phase retardation produces images based mainly on index of refraction. We have developed a microscope that forms images that depend on small differences in extinction for different forms of incident polarized light. By modulating the polarization of light incident on the sample and digitally recording the difference in intensities of transmitted light, we obtain images which specifically reveal either ordered linear structures or chiral (right- or left-handed) structures. Linearly polarized light, incident alternately with two perpendicular directions of polarization, forms images of structures which have linear order or linear orientation. Right-handed and left-handed circularly polarized light incident alternately on a sample forms images of chiral structures. Structures with neither linear order nor chirality are essentially invisible. Thus, images based on linear dichroism, circular dichroism, and linear and circular differential scattering can be used to detect specific types of structures which may be difficult to observe by conventional methods. We have used such 'linear and circular differential imaging' to study the structure of the nucleolus (the site of RNA synthesis) in live primary spermatocytes of *Drosophila* when they are transcriptionally active or inactive. Some inactive nucleoli are bipartite, with two distinct structures visible by differential scattering of both linearly and circularly polarized light. The active nucleolus is a single domain; it is clearly distinguished from part of the Y chromosome, and it shows different internal structure with linearly and circularly polarized light. Thus, polarization-dependent images reveal structures which can be associated with the transcriptional activity of cells.

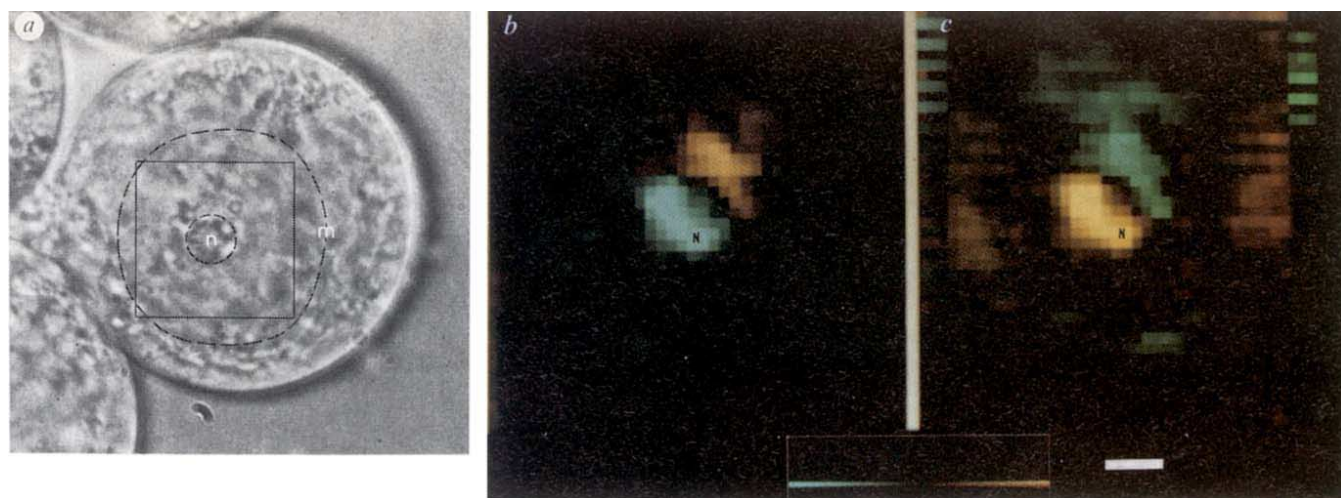
This type of microscopy has several special capabilities. It is possible to vary wavelength, state of polarization, and spatial resolution to probe different cellular components and to produce images sensitive to short- or long-range order for both linear and chiral structures (such as helices). Simple absorption and phase effects (birefringence) are not imaged with this method. No staining is required; in fact, as the images in this study are mainly caused by scattered light, there is little light absorption to damage the cells. In addition, weak reflection polarization from surfaces can be used to determine the shape of the structure being imaged. This microscope has a sensitivity of four parts in one hundred thousand for linear and circular differential extinction. Because the ratio of linear dichroism to absorption is of the order of one for many materials (such as DNA, fibrous proteins and haem-containing proteins), a very small fraction (<0.01%) of oriented material can be detected in the presence of a large amount of randomly-oriented material. Linear dichroism imaging has been used to measure the amount of polymerized haemoglobin in sickle cells<sup>1</sup>. The ratio of circular dichroism to absorption is in general much lower than that for linear dichroism; this gives a lower detection capability for chiral arrangements<sup>2</sup>.

The scanning-stage differential polarization microscope uses a monochromatic light source whose polarization is sinusoidally modulated, and a photomultiplier as a detector (Fig. 1). The output of the detector is calibrated with a polarizer as a linear



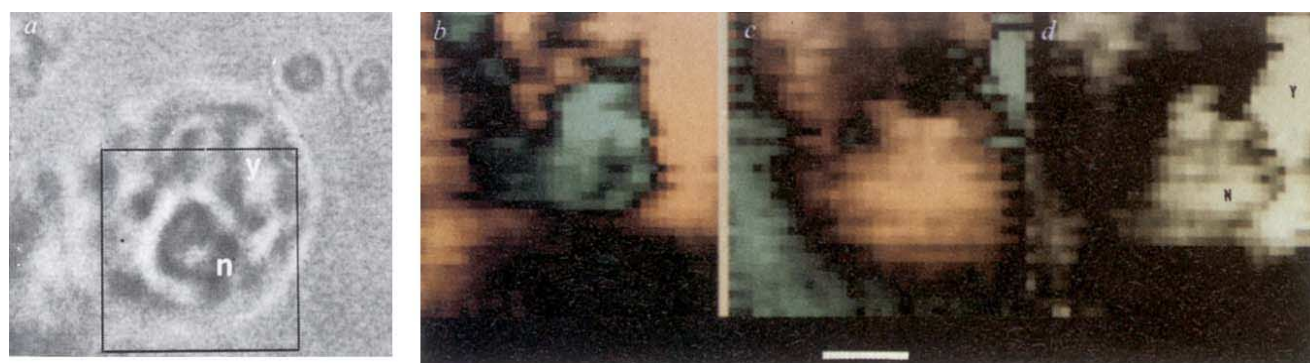
**Fig. 1** Diagram of the scanning-stage differential polarization microscope. A mercury lamp is focused through an interference filter (430 nm, 10-nm bandwidth) onto a pinhole, giving a monochromatic point source. This beam is polarized and its polarization is sinusoidally modulated with a photoelastic phase modulator. The modulated beam is focused on the sample with a  $\times 100$  (numerical aperture 1.25, Zeiss Ultrafluor) polarization-retaining objective. The transmitted beam is focused by another  $\times 100$  objective on another pinhole before the photomultiplier tube (PMT). This pinhole (0.25 mm) is twice the size of the first so that the microscope operates with incoherent imaging. The output of the PMT is used in a feedback loop to keep the photomultiplier output current constant. The PMT output is further split between two lock-in amplifiers, one of which is phase-locked to the polarization modulation and the other is phase-locked to twice the frequency. The amplitude of the photoelastic modulator is adjusted so that the signal at the modulation frequency is primarily circular dichroism; at twice that frequency it is primarily linear dichroism<sup>1,3</sup>. Because of the feedback loop the output of each lock-in amplifier is proportional to the differences in intensities of light transmitted for each polarization ( $I_1$  and  $I_2$ ) divided by the transmitted intensity for both polarizations. This measure is used to determine the differential extinction for that signal as shown below.  $(I_1 - I_2)/(I_1 + I_2) = \tanh(2.303(A_1 - A_2)/2)$ . Here  $A_1 - A_2 = \Delta A$  is the difference in absorbance between the two polarization forms of light. The sensitivity to both linear and circular dichroism at each lock-in amplifier is determined using standards. The results are used to separate analytically the circular and linear differential effects<sup>3</sup>. This information is recorded as the sample is scanned across the beam using a Burleigh micro-positioning stage with an accuracy of 100 nm; about 45 min was required to obtain a complete image. To remove the angular dependence of the linear differential effects, two linear differential images must be taken<sup>1</sup>. These two images differ by a  $45^\circ$  rotation of the two linear polarization forms. The norm of these two images is proportional to the amount of linear differential material; the orientation of the linear material can also be calculated from the images.

dichroism standard and with a set of circular dichroism standards. Linear and circular differential extinctions are recorded<sup>3</sup> as the object is scanned across the light beam. The resolution is defined by the numerical aperture of the lenses, the sizes of pinholes—one before the polarization modulator and the other before the photomultiplier tube<sup>4</sup>—and the scanning step size. Thick samples might have been expected to cause mixing and



**Fig. 2** Three views (*a*, transmission image; *b*, circular differential image; *c*, linear differential image) of the nucleus of a late growth stage primary spermatocyte; many such cells were imaged. All imaging was done with live intact cells. *a*, The transmission image of a representative late primary spermatocyte is shown for comparison with the differential images. Two circles outline the nucleolus (*n*) and the nucleus (*m*, nuclear membrane); a square shows the area of the nucleus corresponding to the differential images. The differential images (*b*, *c*) show the bipartite nature of some late-stage nucleoli. The inset shows the colour bar giving the assignment of colour to differential extinction. The field of view in the differential polarization images is  $14\ \mu\text{m}$  on each side; white scale bar,  $2\ \mu\text{m}$ . The transmission image (*a*) is at half this magnification to show the entire cell. *b*, The circular differential image at  $430\ \text{nm}$  of the nucleus of a late primary spermatocyte. This image shows the two domains of the nucleolus. Circular differential extinction is colour coded with shades of blue representing  $\Delta A = +0.002$  to  $0.0$ ; yellow is coded from  $0.0$  to  $-0.002$ . *c*, The linear differential image of the same two-domain nucleolus (blue,  $+0.002$  to  $0.0$ ; yellow,  $0.0$  to  $-0.002$ ). The change in sign shows a difference in orientation of the material that gives linear differential extinction from each half of the nucleolus. A minor contribution from reflection polarization at the edges of the nucleus is visible in some linear differential images.

**Methods.** Testes from third instar larvae (yellow/white colouration) were dissected, placed in *Drosophila* Ringer's and then gently squashed between two quartz cover slips. Staging was done from the size of cells and from their cytoplasmic constituents. Cytoplasmic streaming was still visible after imaging, indicating the viability of the cells. Trypan blue was excluded from intact cells in such squashes.



**Fig. 3** Four views of the nucleus of an early stage primary spermatocyte; more than ten such cells were imaged. The morphology of this cell is as expected: most of the cytoplasm is contained in a small region still attached to part of the cyst to the left and the nucleus with its prominent nucleolus takes up the remainder of the cell. *n*, Nucleolus; *Y*, presumed Y chromosome. *a*, Transmission image of a primary spermatocyte. Square, area shown in the differential polarization images. The field of view in the differential images (*b*–*d*) is  $7\ \mu\text{m}$  on each side; the wavelength used to image is  $430\ \text{nm}$ . The white bar under the differential images is  $2\ \mu\text{m}$  long. Both linear and circular differential extinction are colour coded with blue representing  $\Delta A = +0.002$  to  $0.0$ ; yellow,  $0.0$  to  $-0.002$ . *b*, Circular differential image showing the nucleolus and part of the Y chromosome. *c*, Linear differential image of the same area as in *b* showing the single domain nucleolus (centre, yellow) and part of the lampbrush chromosome (upper right, blue). *d*, Image showing the amount of linearly aligned material. The image is obtained from two linear dichroism images (one is shown in Fig. 3*c*) with the principal axes of polarization differing by  $45^\circ$ . The norm of these two images gives the amount of oriented material independent of the direction of orientation<sup>1</sup>. The nucleolus and part of the lampbrush chromosome are readily visible in *a*–*d*. Note that the circular and linear differential images show different structures in the nucleolus in this stage of development.

interference of light rays from different regions of the sample, thus giving spurious images. However, we have observed that defocus simply attenuates the signal. This is because of the shallow depth of focus we obtain by working near the confocal imaging condition. The scanning-stage differential polarization microscope combines the photometric accuracy of a scanning-stage microspectrophotometer with a single-beam measurement of linear and circular extinction. With the simple addition of more phase modulators and an analyser, images can be produced

using each of the sixteen components of the Mueller matrix<sup>5</sup> to obtain all possible information on the interaction of light with matter as described by the Mueller optical calculus.

We studied nuclei at two readily identifiable stages in the development of the sperm of the fruit fly *Drosophila melanogaster*. The late growth phase of the primary spermatocyte is characterized by low transcriptional activity<sup>6</sup>, high translational activity<sup>7</sup> and large cell size<sup>8</sup>. The early primary spermatocyte and the morphologically very similar spermatogenic



cell are characterized by high transcriptional activity<sup>7</sup>, low translational activity<sup>8</sup>, smaller size<sup>8</sup> and the early primary spermatocyte contains a lampbrush chromosome<sup>9</sup>.

The difference between the differential images of the nuclei in these two metabolic states is quite dramatic. The transmission image of the late primary spermatocyte shows a single nucleolus in the nucleus (Fig. 2a). The nucleolus is bipartite, as shown in the linear and circular differential images (Fig. 2b and c). The different signs of the linear differential images (Fig. 2c) show a difference in the orientation in each half. The domains seen in the circular differential image may be due to the orientation of the chromosomes or to two different structures. Perhaps the different regions are in different stages of being dispersed and transcriptionally inactivated, which would explain the variation found in the images of nuclei in this stage. This contrasts sharply with what is seen in the cells from the earlier stage. The bipartite nucleolus has only been seen previously in electron microscope studies and may be due to the different chromosomes that make up the nucleolus<sup>10</sup>.

The early-stage cells (Fig. 3) are transcriptionally very active in the nucleolus as well as in the loops of the lampbrush chromosome. Both the circular dichroism image (Fig. 3b) and the image of linearly aligned material (Fig. 3d) show a single-domain nucleolus with what is probably part of the Y chromosome (on its right in Fig. 3). Note that the structure of the aligned material seen in the linear differential images (Fig. 3c and d) is different from the chiral structure seen in the circular differential image (Fig. 3b) of the nucleolus. The largest amount of linearly oriented material (under the N in Fig. 3d) corresponds to a minimum in the signal from the chiral material. These different structures may coincide with the granular and fibrillar regions of the nucleolus<sup>11</sup>. These images show that the presumed granular region (a region of maturation of ribosomes) is arranged in a specific chiral arrangement not seen before. There are other nuclear structures that are not visible in the usual transmission images, but are found in the differential images; these are probably the lampbrush chromosome. The orientation of the aligned material calculated from two linear dichroism images (one of which is shown in Fig. 3c) shows that the lampbrush chromosome is independent of the nucleolar structure. This Y chromosome is divided into actively transcribing regions (the brush of the lampbrush) and the heterochromatic condensed region. We assume that the heterochromatic region which is not transcriptionally active is the large structure to the right of the nucleolus (in Fig. 3d). This has been verified by using a hybrid fly lacking the Y chromosome (X/0). No nuclear structure similar to the lampbrush chromosome is seen in these early primary spermatocytes. This structure has a linear arrangement of scattering material as well as a chiral arrangement; further studies may reveal structural details, such as a helical repeat.

Differential polarization imaging can separate differential absorption, differential scattering and phase effects which are combined in phase and polarization microscopy. In spermatocytes both linear and chiral organization were seen and were found to change as a function of transcriptional activity. The determination of the differential scattering patterns and the differential extinction (linear and circular dichroism) of microscopic samples can give new structural information. Inherent chromophores such as nucleic acids, haems and proteins can be used in live, unstained cells. Dyes that mimic the linear and circular differential extinction of the structure to which they are bound to can be used to image separately nucleic acid<sup>12</sup> and protein structures. Polarization and wavelength conditions can be chosen to give images that are related to specific long-range chiral and linear structures. We found that these images, and therefore the structures that are imaged, change in the developmental sequence of the sperm of *Drosophila*. Both chiral (probably helical) and linear structures are significantly different depending on the metabolic stage of the nucleolus. The exact

structures that give these types of scattering remain to be determined.

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## Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes

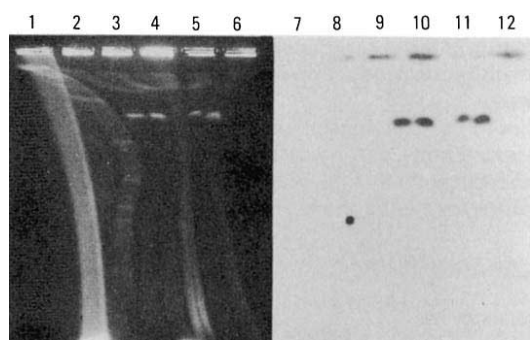
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A number of examples of circular plasmids with specific functions are known in both prokaryotes and eukaryotes. Several linear plasmids have also been identified<sup>1-6</sup>, but these are all relatively small: large linear plasmids cannot be separated from chromosomal DNA by conventional techniques. There are several cases where the genetic evidence suggests that a character is encoded by a plasmid but no plasmid can be physically detected. This has been the case for antibiotic synthesis genes in *Streptomyces*; in particular a plasmid SCP1 in *Streptomyces coelicolor* has been shown to be involved in methylenomycin production by genetic evidence<sup>7,8</sup>. We report here the application of orthogonal-field-alternation gel electrophoresis<sup>9</sup> to the isolation of linear plasmids from *Streptomyces*. We have discovered a large linear plasmid of around 520 kilobases in *Streptomyces lasaliensis* and subsequently similar giant linear plasmids in other *Streptomyces* strains. We have confirmed that genes for methylenomycin biosynthesis<sup>10,11</sup> are located on a series of giant linear plasmids in *S. coelicolor*. These observations may bear on the genetic variability and unstable genetic character of *Streptomyces* species.

In our study of lasalocid biosynthesis in *S. lasaliensis*, we found that protoplast fusion of a producer, UV3-13rif and a non-producer, PR2-45 caused a high frequency transfer of the ability to produce lasalocid<sup>12</sup> and echinomycin<sup>13</sup> (Las<sup>+</sup> Ech<sup>+</sup> phenotype) from UV3-13rif to PR2-45 (K.H., S.L. Otten, J. S. Duncan and C. R. Hutchinson, manuscript in preparation). Although the involvement of a transferable DNA element was suggested by this, we could not find any plasmid by CsCl-ethidium bromide (EtBr) ultracentrifugation and conventional agarose gel electrophoresis. Therefore, to detect a plasmid, we tried orthogonal-field-alternation gel electrophoresis (OFAGE) recently developed for separating large linear DNAs such as yeast chromosomes<sup>9</sup>.

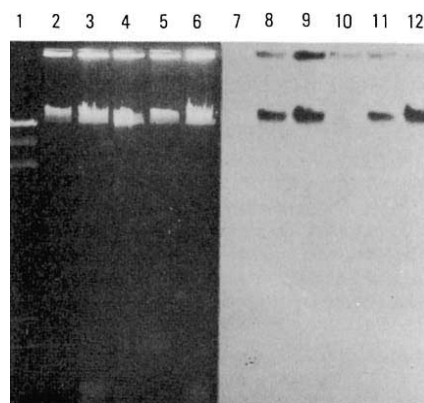
OFAGE analysis clearly revealed a plasmid band in all producers (NRRL3382R, UV3-13rif and Fusants 1 and 2) but not



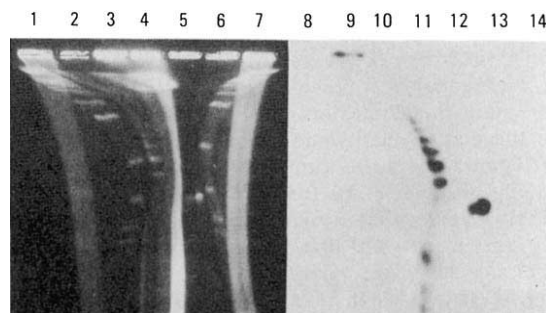
**Fig. 1** OFAGE (lanes 1-6) and Southern blot analysis (lanes 7-12) of *S. lasaliensis* DNAs. Lanes 1, 7, *Saccharomyces cerevisiae* YNN27; lanes 2, 8, *S. lasaliensis* NRRL3382R (Las<sup>+</sup> Ech<sup>+</sup> Nic<sup>+</sup> Rif<sup>s</sup> Str<sup>s</sup>, wild-type strain); lanes 3, 9, UV3-13rif (Las<sup>+</sup> Ech<sup>+</sup> Nic<sup>-</sup> Rif<sup>r</sup> Str<sup>s</sup>, rifampicin-resistant auxotrophic mutant of NRRL3382R); lane 4, 10, PR2-45 (Las<sup>-</sup> Ech<sup>-</sup> Nic<sup>+</sup> Rif<sup>s</sup> Str<sup>r</sup>, streptomycin-resistant non-producer); lanes 5, 11, fusant 1 (Las<sup>+</sup> Ech<sup>+</sup> Nic<sup>+</sup> Rif<sup>s</sup> Str<sup>r</sup>, fusant of NRRL3382R and PR2-45); lanes 6, 12, fusant 2 (Las<sup>+</sup> Ech<sup>+</sup> Nic<sup>+</sup> Rif<sup>s</sup> Str<sup>r</sup>, fusant of UV3-13rif and PR2-45).

**Methods.** Details of mutagenesis and protoplast fusion will be described elsewhere (H.K., S. L. Otten, J. S. Duncan and C. R. Hutchinson, in preparation). *S. lasaliensis* strains were cultured in tryptic soy broth (Difco) containing 0.5% glycine at 30 °C for about three days. Protoplasts, prepared as described in ref. 23, were filtered through glass wool, washed twice with PWP 70 mM NaCl, 10 mM MgCl<sub>2</sub>, 20 mM CaCl<sub>2</sub>, 0.5 M sucrose, 25 mM *N*-tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid, pH 7.2 buffer<sup>23</sup>, and suspended in a mixture of 0.5 ml of 0.5 M sucrose in TSE (30 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA) and 0.1 ml of 0.5 M EDTA (pH 8.0). Then 1.2 ml of 1.5% low-melting-point agarose in 0.5 M sucrose in TSE kept at 37 °C was added and mixed. After solidification of the agarose, 0.5 ml of 5 mg ml<sup>-1</sup> pronase (Actinase E, Kaken-Seiyaku, Tokyo) in TSE and 0.5 ml of 10% SDS were overlaid, and the plate sealed with Parafilm and incubated at 37 °C overnight. The pronase-SDS solution was withdrawn and replaced with 0.5 M EDTA (pH 8.0) and the plate stored at 4 °C. A small piece (1 × 3 × 10 mm) was cut out from the sample gel and applied to a 1.5% agarose gel (10 cm × 10 cm) in 0.5 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The gel apparatus was designed according to Carle and Olson<sup>9</sup> with a modification in which the buffer was kept at 14 °C by circulating cooling water under the gel box instead of circulating the buffer itself through a cooling unit. Electrophoresis was conducted in 0.5 × TBE at 300 V for 26 h with a switching time of 30 s. After electrophoresis, the loading wells of the gel were sealed with 1% agarose in 0.5 × TBE to prevent the removal of the loaded sample gels during the following staining and washing. The gel was stained in 1 µg ml<sup>-1</sup> of ethidium bromide (EtBr) for 20 min and washed in water overnight with shaking. For Southern hybridization, pKSL was electroeluted from the plasmid band on the OFAGE gel, labelled by the Multiprime DNA labelling system (Amersham) and purified on a Sephadex G25 column. Conditions for DNA transfer from the OFAGE gel to nitrocellulose filter and hybridization without formamide were as described by Hopwood *et al.*<sup>24</sup> Yeast chromosomal DNA was prepared from *Saccharomyces cerevisiae* YNN27 by the method of Carle and Olson<sup>9</sup> and used as size standards.

in a non-producer, PR2-45 (Fig. 1). DNA samples were prepared by embedding protoplasts in low-melting-point agarose gel followed by treating with pronase and SDS in the gel to prevent physical shearing of DNA. DNAs prepared by the conventional method showed no plasmid band, showing that this plasmid is broken up during the usual extraction procedure (data not shown). The identity of each plasmid was proved by Southern blotting and hybridization with plasmid pKSL from NRRL3382R as a probe. This probe hybridized to all plasmid bands of producers (Fig. 1), which suggested that pKSL was transferred from NRRL3382R or UV3-13rif to PR2-45 by protoplast fusion. Weak hybridization to the origins might be caused



**Fig. 2** Conventional agarose gel electrophoresis (lanes 1-6) and Southern blot analysis (lanes 7-12) of *S. lasaliensis* DNAs. Electrophoresis of the same sample gels as in Fig. 1 was carried out with 0.7% agarose in 1 × TBE at 40 V for 16 h. Hybridization was performed as in Fig. 1. Lane 1, 7,  $\lambda$  DNA digested with *Hind*III; lanes 2, 8, *S. lasaliensis* NRRL3382R; lanes 3, 9, UV3-13rif; lanes 4, 10, PR2-45; lanes 5, 11, fusant 1; lanes 6, 12, fusant 2.



**Fig. 3** OFAGE (lanes 1-7) and Southern blot analysis (lanes 8-14) of *S. coelicolor* DNAs. Lanes 1, 7, 8, 14, *S. cerevisiae* YNN27; lanes 2, 9, *S. coelicolor* A3(2) (SCP1<sup>+</sup> SCP2<sup>+</sup>); lanes 3, 10, M124 (*proA1 argA1 cysD18 SCP1<sup>-</sup> SCP2<sup>-</sup>*); lanes 4, 11, M130 (*hisA1 uraA1 strA1 SCP1<sup>-</sup> SCP2<sup>-</sup>*); lanes 5, 12, M138 (*proA1 argA1 cysD18 SCP1<sup>+</sup> SCP2<sup>-</sup>*); lanes 6, 13, M146 (*hisA1 uraA1 strA1 SCP1<sup>+</sup> SCP2<sup>-</sup>*). *S. coelicolor* strains were grown in YEME medium (ref. 24) and DNAs were prepared as in Fig. 1. Electrophoresis was at 300 V for 28 h with a 30-s switching time. Southern blot analysis was carried out as in Fig. 1 with nick-translated <sup>32</sup>P-labelled pIJ601 as a probe.

by contamination of degraded chromosomal DNA in the probe preparation, which in turn hybridized to intact chromosomal DNAs remained in the loading wells. The linearity of pKSL was revealed as follows. First, circular DNA moves much more slowly than circular DNA on the OFAGE gel; for example, covalently closed circular pBR322, of 4.3 kilobases (kb) migrates as a linear DNA of 350 kb in the conditions of Fig. 1. Because no small circular-plasmid band was seen on a conventional agarose gel (Fig. 2), pKSL was proved not to be circular. Second, pKSL remained in the same position relative to yeast linear chromosomes under different switching conditions, which clearly shows that it moved as linear DNA (data not shown). The size of pKSL was determined to be 520 kb, as it migrated at a rate intermediate between those of yeast chromosomes IX (460 kb) and VIII or V (580 kb)<sup>14</sup>.

The same sample gels were also subjected to conventional agarose gel electrophoresis. All large linear DNAs such as linearized or degraded chromosomal DNA and intact or degraded pKSL comigrated to form a 'chromosomal DNA' band on the conventional gel, whereas intact circular chromosomal



**Fig. 4** Southern blot analysis of *Pst*I fragments of *S. coelicolor* plasmids. Lanes 1, pIJ601; lanes 2, SCP1(410 kb); lanes 3, SCP1(440 kb); lanes 4, SCP1(470 kb); lanes 5, pM138; lanes 6, pM146. Plasmids, SCP1s(410, 440 and 470 kb), pM138 and pM146 were isolated from *S. coelicolor* A3(2), M138 and M146, respectively as in Fig. 1. These plasmids and pIJ601 were digested with *Pst*I and subjected to conventional agarose gel electrophoresis and Southern blot analysis with <sup>32</sup>P-labelled pIJ601 as a probe.



DNA remained at the origin. Southern hybridization analysis confirmed this interpretation; <sup>32</sup>P-labelled pKSL hybridized to all chromosomal DNA bands but not to that of PR2-45. On the other hand, contaminating degraded chromosomal DNA in the probe hybridized weakly to all intact chromosomal DNAs remaining at the origin. These results clearly indicate that large linear DNA cannot be separated from degraded chromosomal DNA in this system.

That the presence of pKSL is related to antibiotic production in *S. lasaliensis* suggests that this plasmid is involved in the production of lasalocid and/or echinomycin. We further searched for and detected giant linear plasmids from five other strains in which antibiotic production is postulated to be plasmid determined including *S. coelicolor* A3(2) (ref. 15).

Next, we tried to test for localization of antibiotic biosynthetic genes on giant linear plasmids using the *S. coelicolor* system, because the cloned methylenomycin biosynthetic (*mmr*) and resistant (*mmr*) genes are known to form a gene cluster on a unisolatable plasmid, SCP1 (ref. 13). OFAGE analysis (Fig. 3) surprisingly revealed that the wild-type strain, *S. coelicolor* A3(2) contains a series of giant linear plasmids (410–560 kb) with a size difference of 30 kb, whereas two other SCP1<sup>+</sup> strains, *S. coelicolor* M138 and M146 have only one plasmid of 350 kb. On the other hand, the two SCP1<sup>−</sup> strains *S. coelicolor* M124 and M130 have no plasmid: Probe pIJ601, which contains the *mmr* gene, hybridized to all plasmid bands regardless of their lengths. Furthermore, *Pst*I digestion revealed that all plasmids contained the same 2.6-kb *mmr* fragment (Fig. 4); the larger fragment in lane 5 might be formed by partial digestion of the plasmid. These results demonstrate that both a series of giant linear plasmids in *S. coelicolor* A3(2) and a 350-kb plasmid in *S. coelicolor* M138 and M146 are SCP1 itself. Variation of SCP1 in *S. coelicolor* A3(2) might be formed by multiplication of a 30-kb segment of a 350-kb plasmid, a possibility which should be studied further. We have revealed for the first time the physical identity of SCP1 and confirmed the presence of the methylenomycin biosynthetic genes on it. However, we cannot exclude the possibility that SCP1 is present at the same time in an integrated form in *S. coelicolor* A3(2), because the probe hybridized weakly to the origin of lane 9 in Fig. 3.

We found giant linear plasmids in a total of six antibiotic producing strains of *Streptomyces* by the newly developed OFAGE technique. Hayakawa *et al.*<sup>1</sup> reported a linear plasmid (pSLA2 of 17 kb) from *S. rochei* 7434-AN4, a producer of lankacidins, which has been the only linear plasmid isolated from *Streptomyces*. The sizes of linear plasmids so far isolated, including pSLA2, range from 5.4 to 17 kb. Therefore, the plasmids we describe here are the first examples of giant linear plasmids from prokaryotes or eukaryotes; the size of pKSL (520 kb) is one-twentieth that of chromosomal DNA (10<sup>4</sup> kb)<sup>16</sup>. This new type of genetic structure is very interesting in view of the many unstable genetic characters of *Streptomyces* such as aerial mycelium formation<sup>17</sup>, pigment production<sup>18</sup>, drug resistance<sup>19</sup> and arginine biosynthesis<sup>20</sup> in addition to antibiotic production<sup>21,22</sup>. The relation between a giant linear plasmid and antibiotic production in *S. lasaliensis* is circumstantial, but it

has been confirmed in *S. coelicolor*. The giant linear plasmids may function not only in *S. coelicolor* but also in other strains in which antibiotic production is postulated to be plasmid determined.

We thank C. R. Hutchinson and D. A. Hopwood for providing *S. lasaliensis* strains, and *S. coelicolor* strains and pIJ601, respectively; K. Sakaguchi and M. Kageyama for encouragement and R. Matsuura for typing the manuscript.

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## Corrigendum

### Structure, sequence and expression of the hepatitis delta (δ) viral genome

Kang-Sheng Wang, Qui-Lim Choo, Amy J. Weiner, Jing-Hsiung Ou, Richard C. Najarian, Richard M. Thayer, Guy T. Mullenbach, Katherine J. Denniston, John L. Gerin & Michael Houghton

*Nature* **323**, 508–514 (1986).

A GUANOSINE residue was omitted from the nucleotide sequence of the hepatitis δ viral (HDV) genomic RNA between residues 1,113 and 1,114 (Figs 4 and 5). HDV RNA therefore consists of 1,679 nucleotides and Table 1 should now read as given below.

**Table 1** Large open reading frames (ORFs) within HDV RNA and the complementary anti-HDV RNA strand

| Strand       | ORF no. | Translational frame | Nucleotide position* | Total no. of AAs | No. of AAs beginning with first Met |
|--------------|---------|---------------------|----------------------|------------------|-------------------------------------|
| HDV RNA      | 1       | 2                   | 539                  | 165              | 152                                 |
| HDV RNA      | 2       | 1                   | 786                  | 156              | 121                                 |
| HDV RNA      | 3       | 1                   | 1,608                | 120              | 0                                   |
| HDV RNA      | 4       | 3                   | 1,297                | 115              | 68                                  |
| HDV RNA      | 10      | 1                   | 435                  | 116/39†          | 33                                  |
| HDV RNA      | 11      | 3                   | 937                  | 119              | 86                                  |
| Anti-HDV RNA | 5       | 1                   | 1,619                | 221/202‡         | 214/195‡                            |
| Anti-HDV RNA | 6       | 3                   | 1,341                | 288              | 179/34‡                             |
| Anti-HDV RNA | 7       | 1                   | 506                  | 148/80§          | 148/80§                             |
| Anti-HDV RNA | 8       | 1                   | 821                  | 104/56           | 74/26                               |
| Anti-HDV RNA | 9       | 2                   | 91                   | 101              | 0                                   |

Only ORFs longer than 300 nucleotides are shown. AAs, amino acids.

\* The position of the first nucleotide in each ORF is indicated according to the numbering in the corrected Fig. 4.

† Ambiguity arising from clonal heterogeneity at position 553.

‡ Ambiguity arising from clonal heterogeneity at position 1,012.

§ Ambiguity arising from clonal heterogeneity at position 264.

|| Ambiguity arising from clonal heterogeneity at position 653.

# Catalytic antibodies catching on

R.J Massey

*Designer proteins, accelerated syntheses, and better purification procedures are some of the potential benefits of a melding of the best of antibodies and catalysts.*

CLASSICALLY, antibodies have been characterized as proteins which have evolved with the sole function of binding other molecules, called antigens. Antibodies are distinct from enzymes, that not only bind other molecules specifically, but also have the ability to catalyse chemical reactions. The specific molecular recognition characteristics of antibodies, through the interaction of specific binding sites, have caused scientists to wonder if antibodies have the potential to catalyse chemical reactions, like enzymes.

The key to unlocking the answer to this question can be traced back to Emil Fischer<sup>1</sup>, who developed the concept that the action of enzymes depends on the geometrical structure of the molecule to be attacked, and that the two must match like lock and key. Linus Pauling<sup>2</sup> modified Fischer's lock and key concept to suggest that an enzyme active site should not match the substrate, but rather be complementary to the activated complex, or transition state, of the reaction being catalysed. Pauling also provided the insight for the discovery that antibodies could function like enzymes when he stated that an enzyme has a structure closely similar to that found for antibodies, but with one important difference. Pauling posited that the surface configuration of an enzyme is not as closely complementary to its specific substrate as an antibody is to its antigen, but is instead complementary to the activated complex. William Jenks provided another perspective<sup>3</sup> by suggesting that if complementarity between the active site and transition state contributes significantly to enzyme catalysis, it could be possible to synthesize an enzyme by constructing a binding site. One way to do this would be to prepare an antibody to a haptenic group which resembles the transition state of a given reaction. The combining sites of such antibodies should be complementary to the transition state and should accelerate the reaction rate by forcing the bound substrates to resemble the transition state.

## Early tries

Initial attempts to isolate polyclonal antibodies with catalytic activity were unsuccessful. With the advent of hybridoma technology, the means to produce catalytic monoclonal antibodies was created. In 1985, a method for generating catalytic monoclonal antibodies, and a way to use those antibodies to accelerate chemical reactions, were described by

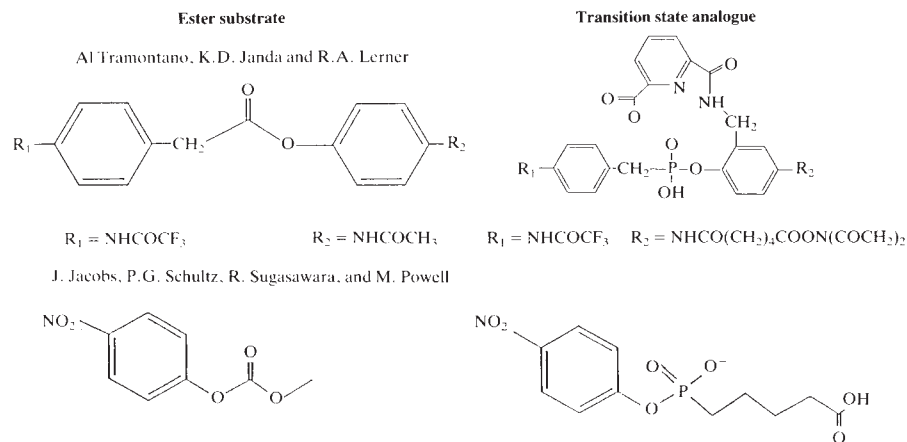


Fig. 1 Substrates and immunogens for catalytic antibodies.

Schochetman and Massey<sup>4</sup>. Recently, several groups using transition state analogues as immunogens have produced monoclonal antibodies that bind the transition state analogue and catalyse a chemical reaction. Tramontano, Janda and Lerner<sup>5</sup> (Fig. 1), and Pollack, Jacobs and Schultz<sup>6</sup> demonstrated that antibodies that bind tetrahedral phosphorous compounds, which mimic the presumed transition state in the hydrolysis of a carboxylate ester, also have catalytic activity.

Tramontano and Lerner demonstrated that monoclonal antibodies generated against a phosphonate ester could catalyse the hydrolysis of analogous carboxylic esters. The  $K_{cat}$  values measured at pH 9 for two ester substrates with the monoclonal antibody 6D4 were  $1.62 \text{ min}^{-1}$  and  $0.48 \text{ min}^{-1}$ , while the corresponding  $K_m$  values were  $1.90 \text{ }\mu\text{M}$  and  $0.62 \text{ }\mu\text{M}$ . Schultz's group tested the known mouse myeloma protein MOPC 167, which binds nitrophenyl phosphorylcholine, as a catalyst for the hydrolysis of an analogous carbamate and measured a  $K_{cat}$  of  $0.4 \text{ min}^{-1}$  and a  $K_m$  of  $208 \text{ }\mu\text{M}$  at pH 7. These rates correspond to accelerations of approximately 1,000-fold over the uncatalysed rates, but are approximately 5,000-fold slower than  $\alpha$ -chymotrypsin acting upon a good ester substrate.

Both research groups noted that their antibodies have many of the same characteristics as enzymes. Catalytic antibodies and enzymes both show substrate specificity, exhibit saturation kinetics, and are subject to competitive inhibition. While the detailed chemical mechanisms of these catalytic antibodies have yet to be determined, it is reasonable to suppose that the planar  $sp^2$  hybridized ester or carbamate is strained towards a tetrahedral geometry

upon binding, thus facilitating the attack of hydroxide ion. Indeed, the antibody-catalysed hydrolyses all show a first order dependence on hydroxide ion concentration. More recently, Schultz's group, in collaboration with Sugasawara and Powell<sup>7</sup>, raised monoclonal antibodies to a tetrahedral, 4-nitrophenyl phosphonate species, a transition-state analogue for the hydrolysis of methyl 4-nitrophenyl carbonate (Fig. 1). A number of antibodies have been isolated that selectively catalyse the hydrolysis of the carbonate, one with a  $K_{cat}$  of  $1.4 \text{ min}^{-1}$  and  $K_m$  of  $660 \text{ }\mu\text{M}$ , and the other with a  $K_{cat}$  of  $29 \text{ min}^{-1}$  and  $K_m$  of  $350 \text{ }\mu\text{M}$ . This represents rate accelerations of 810 and 16,500, respectively, over the uncatalysed reactions.

## Catalytic potential

As an emerging technology, the applications of catalytic antibodies are just beginning to be realized. Protein engineering represents an important frontier in biotechnology, and enzymes produced by recombinant DNA technology have already assumed a prominent position in the marketplace. However, there are a limited number of enzymes to work with, and they do not show the specificity and diversity exhibited by antibodies. The breakthrough that catalytic antibodies provide is the ability to take the guess work out of correlating structure with function, and use a mechanistic approach in designing catalytic functions.

The market potential for designer proteins is large, and the production of catalytic antibodies provides a rational way to design proteins which address these market opportunities. Custom-designed catalytic antibodies will come to be recognized as a viable approach to enzyme



engineering. For instance, antibodies are being developed to catalyse hydrolysis reactions and the formation of peptide bonds with a very high degree of specificity. Amino-acid sequence-specific catalytic antibodies are essentially the equivalent for proteins of DNA restriction enzymes. They would give scientists the ability to cut and paste proteins, and could be enormously important tools for protein study. In addition to providing powerful tools for basic research, such enzymes might find a number of uses in medicine, for selectively cleaving peptide bonds in proteins that form, for example, the coat of a particular virus.

"Semisynthetic" catalytic antibodies, or antibodies that bind a specific molecule, to which catalytic groups such as cofactors or metals are chemically attached, are another possibility. The antibody portion would afford affinities and specificities, while the synthetic catalyst would open up all kinds of unique chemical possibilities. Alternatively, site-directed mutagenesis could be used to engineer amino acid residues that would confer catalytic activity to an antibody.

Stereoselective control of organic chemical reactions, including asymmetric oxidation and reduction reactions, and specific isomerization procedures such as Claisen rearrangements, are being actively investigated with catalytic antibodies. Chiral resolution of high value racemic mixtures, normally performed using crystallization procedures, can be greatly accelerated employing catalytic antibodies as chiral resolving reagents.

Given the exquisite stereoselectivity and specificity of antibody combining sites, the use of novel catalytic antibodies for pharmaceutical synthesis, particularly antibiotic synthesis, is being studied. The hydroxylation of novel pharmaceutical precursors to provide new drugs for clinical evaluation, and to improve current synthesis strategies for difficult to obtain pharmaceuticals, is another possibility.

Considering the number of possible uses for catalytic antibodies, there is much exciting chemistry and immunology to be done. Nature has the ability to generate for us in a matter of months a highly selective binding pocket for almost any molecule of interest. □

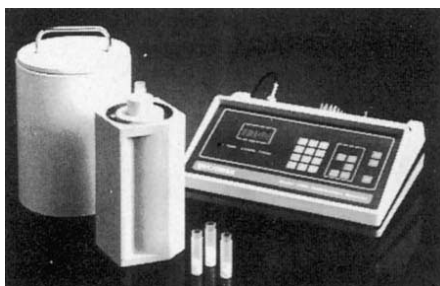
*Richard J. Massey is the vice president of Igen, Inc., 1530 East Jefferson Street, Rockville, Maryland 20852, USA. For more information, fill in reader service number 100.*

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# High technology in recDNA

*Recombinant DNA is the topic for this week, with products of use to every DNA researcher, including kits for cloning, tools for electrophoresis, and off-the-shelf synthetic genes.*

FOR end-labelling, nick translation or hybridization assays where  $^{125}\text{I}$  or  $^{32}\text{P}$  labels are used, Beckman has introduced a new **single sample radioisotope detector** (Reader Service No. 101). The model



*A radioisotope detector for single samples.*

170M measures  $^{125}\text{I}$  with 45 per cent efficiency and  $^{32}\text{P}$  with 20 per cent counting efficiency, says Beckman. The 170M can be used in a cold room, and is compact enough to fit on the benchtop. Beckman's radioisotope detector carries a \$2,750 (US) price tag.

Molecular Biology Resources has a **hybridization apparatus** to simplify the hybridization process (Reader Service No. 102). The apparatus, available in 82 mm and 138 mm diameter sizes, is a minimum volume chamber that MBR says allows steps from pre-hybridization to sequential



*The chamber's window is a beta block.*

buffer wash to be performed in minutes, not days. The apparatus can be submerged in a water bath to control temperature, and can process up to 20 blots at a time. The chamber window acts as a beta block, and the self-cleaning chamber is decontaminated automatically during the buffer wash cycle. MBR's hybridization apparatus costs \$340 and \$395 (US) for

the two sizes. Accessories to convert the apparatus into a slot blotting manifold are also available.

Savant Instruments has put some of the **most-used instruments in DNA work** onto one convenient cart, and has dubbed the resulting system the "Gene Jockey's Delight" (Reader Service No. 103). It consists of a 100-tube Speed-Vac concentrator, a 40 × 50 cm-bed slab gel dryer, a mechanically refrigerated trap and a vacuum pump — all mounted on a two-shelf cart with four electrical outlets. The system concentrates and dries natural and synthetic preparations of DNA, and



*Savant's "Gene Jockey's Delight".*

evaporates organic solvents to permit 100 per cent recovery of the sample, says Savant, for electrophoretic analysis. The system also dries the large slab gels used to separate and identify DNA fragments.

Microquantities of DNA and protein are not minute enough to evade analysis by Perkin-Elmer's DNA QUANT **application system**, for use with the Perkin-Elmer Lambda 4B UV-visible spectrophotometer (Reader Service No. 104). Biological samples as small as 50 µl can be analysed with the \$1,980 (US) system, that consists of a UV Biotech software cartridge and a quartz microcuvette. The system has a variety of applications, including the computation of:  $A_{260}/A_{280}$  ratios, double- and single-stranded DNA, RNA, and oligomer concentrations; and protein and nucleic acid concentrations in simple mixtures. The 50 µl quartz microcuvette is large enough for easy handling, and its open-top design makes it simple to clean.

A guidebook on **methods for working with synthetic DNA** is now available from

Applied Biosystems (*Reader Service No. 105*). Entitled *Evaluation and Purification of Synthetic Oligonucleotides*, the 56-page book discusses standard synthesis methods, and the specific purification problems associated with each chemistry. Chapters on analysis of the crude product, purification techniques (with special emphasis on HPLC), and verification and storage of the purified product are also included. The book, full of data, charts and chromatograms, is free to DNA researchers.

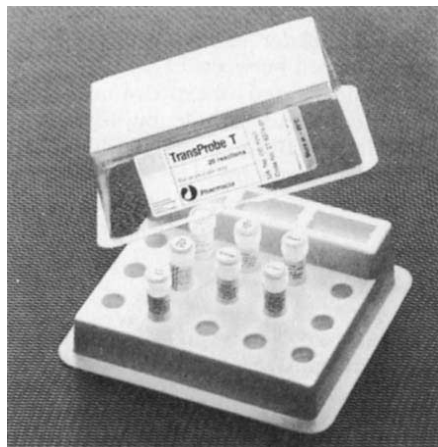
### Probing, purifying and sequencing

5 Prime  $\rightarrow$  3 Prime, Inc. has a **plasmid purification column** designed to provide CsCl gradient purity in less than three hours, without ultracentrifugation (*Reader Service No. 106*). Using the spun columns, DNA can be purified from up to a one litre culture with a 25-minute low-speed centrifugation step. The columns are tested for performance using HB101 transformed with pBR322. Each column comes with collection and processing tubes and a detailed protocol. The columns sell for \$31 (US) for three columns.

Chemiprobe is the name of a **non-radioactive chemical DNA probe labelling kit** from FMC BioProducts (*Reader Service No. 107*). Chemiprobe labels probes by modifying cytosines within the probe DNA, that are in turn recognized by a monoclonal antibody. Visualization of hybridized DNA is achieved using a second enzyme-labelled antibody. The procedure does not employ nick translation enzymes or biotinylation, and can be carried out with unpurified DNA. FMC BioProducts says sensitivities of down to 1–3 pg can be obtained with dot blots of target sequence DNA. The modified probes are

stable for one year at  $-20^{\circ}\text{C}$ . Chemiprobe works on nitrocellulose and most nylon membranes, and one \$210 (US) kit will modify 1 mg of DNA and allow visualization of 1,000  $\text{cm}^2$  of membrane.

Labelled RNA probes or  $m^7\text{GpppG}$  capped RNA for **eukaryotic *in vitro* transcriptions** can be produced with Pharmacia's TransProbe T kit (*Reader Service No. 108*). The kit contains reaction buffer, RNAGuard (to protect RNA transcripts from enzymatic degradation),  $m^7\text{GpppG}$  cap structure, FPLC-pure RNase-free DNase 1, RNase-free water, and FPLC-pure T3 and T7 RNA polymerase. The reaction is completed in a single test tube, and yields microgram quantities of RNA at a specific activity of approximately  $1 \times 10^9$  d.p.m.  $\mu\text{g}^{-1}$ , according to Pharmacia. The resulting transcripts are biologically active in eukaryotic translation systems

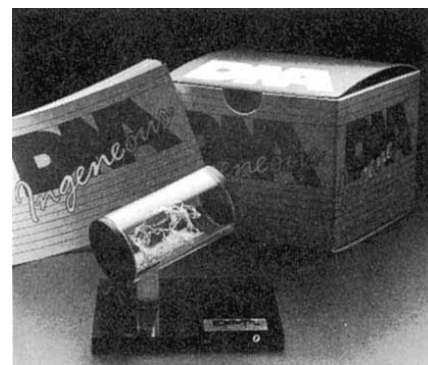


Pharmacia's kit for capped RNA.

such as wheat germ, *Xenopus laevis* oocytes or reticulocyte lysates. Each kit performs 20 reactions. A TransProbe kit using SP6 RNA polymerase is also available.

For the construction of **controlled unidirectional deletions**, Promega offers its Erase-a-Base system (*Reader Service No. 109*). The system is designed to facilitate dideoxy sequence analysis of large DNA fragments cloned in plasmid or M13 vectors, and is based on the method developed by Henikoff (*Gene* **28**, 351; 1984). According to Promega, starting with appropriately treated recombinant DNA, a collection of subclones containing stepwise deletions spanning several kilobases can be constructed in several hours. Extraction and precipitation of multiple samples is unnecessary, because all manipulations are performed by successive additions of the various reagents. The Erase-a-Base system contains enough reagents for 50 reactions consisting of 25 time points, and costs \$198 (US).

Boehringer Mannheim Biochemicals has a new **site-directed mutagenesis kit** (*Reader Service No. 110*). The kit is based

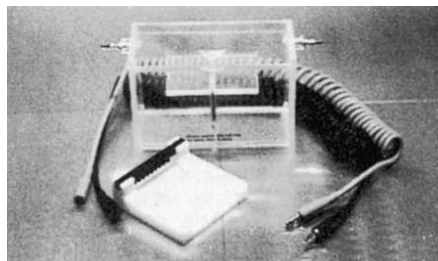


To help DNA researchers explain their work to friends and associates in a tangible way, Tyler Research Instruments Ltd has devised a unique gift for its clients — a desk sculpture/paperweight composed of a vial containing a lacy dehydrated isolate of purified DNA (*Reader Service No. 118*). The sculpture, entitled *Ingeneous DNA*, comes with a booklet to acquaint laymen with the concept that genes are composed of DNA, and that DNA is the blueprint for the synthesis of proteins, from which all life is made. The booklet delves into and demystifies common genetic engineering techniques, and even defines the uses of plasmids and restriction enzymes. Tyler has had such a healthy response from recipients of the sculptures, that it is now selling them for \$20 (Canada).

on the gapped-duplex method of W. Kramer *et al.* (*Nucleic Acids Res.* **12**, 9441; 1984), and eliminates the primer displacement, nucleotide misincorporation and secondary structure inhibition problems normally associated with *in vitro* mutagenesis, says Boehringer Mannheim. The company claims experimental results with up to 90 per cent efficiencies are possible, and guarantees a mutation frequency of greater than 70 per cent using its control reactions. The kit comes with reagents for 25 experimental and five control reactions including vectors, strains, Klenow, T4 DNA ligase, nucleotides, buffer and protocol.

### Electrophoresis notes

Mini-lysates and restriction digests are just the right size for Universal Bio-Systems' **Pee-Wee agarose gel apparatus** (*Reader Service No. 111*). Using the apparatus, gels can be run at 125 V, under constant voltage and with a Tris-acetate



The Pee Wee agarose gel apparatus solves "small" problems.

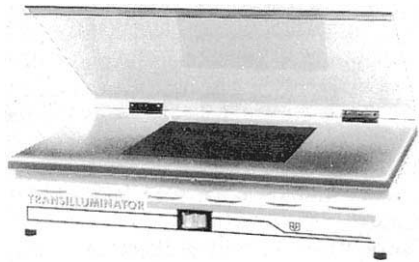


Chemiprobe labels probes without radioactivity.



buffer, in 30 to 40 minutes, according to the company. The \$185 (US) unit consists of two coiled power leads, a gel casting tray, five 50 × 75 mm microscope/gel casting slides, a sliding lid and a choice of analytical or preparative combs. The buffer capacity is 375 ml. The gel table is designed to keep the agarose pillow from drifting horizontally during electrophoresis.

For the **illumination of fluorescently stained gels**, Ultra-Violet Products has a

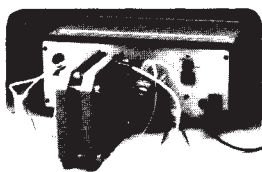


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designed to shield the operator from exposure to harmful UV rays, and to protect the filter frame from accidental damage. The range includes ten versions, that can irradiate gels from mini-size to 40 cm, with 254, 302 or 365 nm rays. The company says the transilluminators provide high-intensity UV irradiance over the full viewing area, creating an even distribution of UV fluorescence over the gel from border to border. Ultra-Violet Products includes a free gel cutter in the transilluminators' £357–1,150 (UK) purchase prices.

### Reagents and molecules

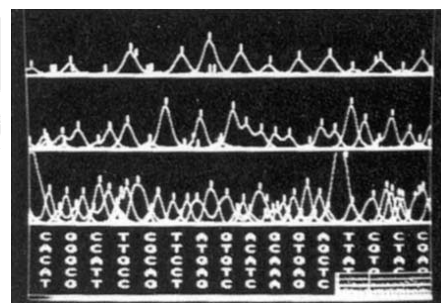
Complete, **double-stranded genes** for important biological proteins can now be purchased through a catalogue. British Bio-technology Ltd has introduced six synthetic human genes, for interleukin 1 alpha, interleukin 2 beta, interleukin 2, tumour necrosis factor alpha, tumour necrosis factor beta, and interferon gamma (Reader Service No. 113). The genes, priced between £1,284 and £1,626 (UK), are created for expression in *Escherichia coli*, and include built-in cutting sites. British Bio-technology Ltd plans to expand the range by 20 to 30 new genes by the end of 1987, including genes for interleukin 3, epidermal growth factor, insulin-like growth factor 1 and horseradish peroxidase.

Pre-mixed, pre-weighed **buffer for Western transfer** is available from Hoefer Scientific Instruments, under the brand name Transphor (Reader Service No. 114). The Transphor mix is available in packs of ten for \$35 (US). Each package makes four litres of buffer. The buffer is easy to prepare: the mix is combined with 800 ml of methanol in a 4 l container, and distilled water is added until the container is full. The final composition of the buffer is 0.025 M Tris, 0.0192 M glycine and 20 per cent methanol, with a pH of 8.3.

A less-expensive **substitute for <sup>35</sup>S methionine** is available from ICN Bio-medicals (Reader Service No. 115). ICN's Tran <sup>35</sup>S-Label is 85 per cent <sup>35</sup>S methionine, and 15 per cent <sup>35</sup>S cysteine, and the company says it has been proven in field tests to be equivalent to <sup>35</sup>S methionine when used to label mammalian cells in culture and proteins *in vivo*. Tran <sup>35</sup>S-Label is derived from <sup>35</sup>S *E. coli* hydrolysate, at a specific activity of >1,000 Ci mmol<sup>-1</sup>. It is provided in an aqueous solution containing 10 mM 2-mercaptoethanol and packaged in a resealable vial at 10 mCi ml<sup>-1</sup> for \$175 (US).

### Genetics-on-line

Bio-Rad has taken another step forward in DNA sequencing by adding an **automated scanner** to its Gene-Master DNA workstation (Reader Service No. 116). The Gene-Master's digitizing pen has



A sample screen display from Gene-Master.

been replaced with an optical scanner based on a linear CCD array detector. The system is completed with pattern recognition and sequence assignment software, and an IBM AT or compatible computer. Comprehensive multi-tasking software for shotgun overlapping, sequence analysis and database searching is also included. Bio-Rad says the automatic reader is able to process conventional autoradiograms with 24-hour operation, with accuracy equal to a human expert reader. The system provides an autoradiogram record for verification, uses commonly available reagents, and supports both Sanger and Maxam-Gilbert chemistries. The scanner is part of a \$48,500 (US) Gene-Master system.

LKB Instruments has a **DNA sequence analysis program** manufactured by Hitachi, DNASIS, that it says can find every site for 100 restriction enzymes on pBR344 in just minutes (Reader Service No. 117). DNASIS is equipped with routines for listing, manipulating, translating and searching, and can handle sequences up to 63,000 nucleotides in length. Data can be entered from the keyboard, or directly from an autoradiograph with a sonic digitizer, and an optional speech synthesizer provides fool-proof recording. DNASIS presents open reading frames, determines codon frequencies, finds sequence regions rich in specified residues, predicts secondary structures and locates palindromes and hairpin loops. It can generate restriction maps automatically from gel data. DNASIS can interface with a CD-ROM disk drive for instant access to GenBank and NBRF sequence data, stored on a single laser disk. DNASIS costs \$1,600 (US).

### Setting it straight

The review of a set of multiple-labelling protocols from Vector Laboratories, published in the 4 June issue of *Nature* (327, p. 445) contained a picture of a slide that was mislabelled. The slide depicted a tissue section labelled with Vector's fluorescent reagents. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.